Free Thyroxine Concentrations by Equilibrium Dialysis and Chemiluminescent Immunoassays in 13 Hypothyroid Dogs Positive for Thyroglobulin Antibody

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Objective: To determine if concentrations of free thyroxine (FT4) measured by semi-automated chemiluminescent immunoassay (CLIA) correspond to FT4 determined by equilibrium dialysis (ED) in hypothyroid dogs positive for thyroglobulin antibody (TGA).

Animals: Thirteen TGA-positive dogs classified as hypothyroid based on subnormal FT4 concentrations by ED.

Methods: Qualitative assessment of canine TGA was performed using an enzyme-linked immunosorbent assay. Serum total thyroxine and total triiodothyronine concentrations were measured by radioimmunoassay. Serum FT4 concentration was determined by ED, and also by semi-automated CLIA for human FT4 (FT4h) and veterinary FT4 (FT4v). Canine thyroid stimulating hormone concentration was measured by semi-automated CLIA.

Results: Each dog's comprehensive thyroid profile supported a diagnosis of hypothyroidism. For detection of hypothyroidism, sensitivities of CLIA for FT4h and FT4v were 62% (95% CI, 32–85%) and 75% (95% CI, 36–96%), respectively, compared to FT4 by ED. Five of 13 (38%) dogs had FT4h and 2 of 8 (25%) dogs had FT4v concentrations by CLIA that were increased or within the reference range. Percentage of false-negative test results for FT4 by CLIA compared to ED was significantly ($P < .0001$ for FT4h and $P < .001$ for FT4v) higher than the hypothesized false-negative rate of 0%.

Conclusions and Clinical Importance: Caution should be exercised in screening dogs for hypothyroidism using FT4 measured by CLIA alone. Some (25–38%) TGA-positive hypothyroid dogs had FT4 concentrations determined by CLIA that did not support a diagnosis of hypothyroidism.

Key words: chemiluminescence; FT4; hypothyroidism; TGAA.

P rimary hypothyroidism is the most common form of naturally occurring hypothyroidism in adult dogs and is characterized histologically by diffuse lymphocytic infiltration or atrophy of thyroid tissue.[1] Although lymphocytic thyroiditis is considered an immune-mediated disorder, it is unclear if thyroid atrophy represents the end stage of lymphocytic thyroiditis or a separate degenerative process.[1,2] Dogs with primary hypothyroidism typically have subnormal concentrations of total thyroxine (T4) and free T4 (FT4), and 75% have increased concentration of thyroid stimulating hormone (TSH).[1,2] In addition, 36–53% of dogs with hypothyroidism have circulating thyroglobulin antibody (TGA), presumably associated with lymphocytic thyroiditis.[2–5] Of dogs that test positive for TGA, approximately 10% and 30% also have circulating anti-

Abbreviations:

CI	confidence interval
CLIA	chemiluminescent immunoassay
ED	equilibrium dialysis
FT4	free thyroxine
FT4h	human FT4
FT4v	veterinary FT4
QNS	quantity not sufficient
RIA	radioimmunoassay
T3	triiodothyronine
T4	thyroxine
TGA	thyroglobulin antibody
TSH	thyroid stimulating hormone

bodies to T4 and T3, respectively.[2,a] Thyroid hormone antibodies may interfere with thyroid hormone immunoassay measurements causing misleading results.[1,2]

Free T4 is the unbound biologically active portion of total T4. Historically, the optimal methods for measuring FT4 in dogs utilized techniques that isolated FT4 from protein-bound T4 by equilibrium dialysis (ED), or by a specialized 2-step radioimmunoassay (RIA). The FT4 2-step RIA is no longer commercially available. Currently, ED is considered the "gold standard" for determination of FT4 in the dog because interference from circulating thyroid hormone antibodies or other binding proteins is eliminated by dialysis. By contrast, semi-automated chemiluminescent immunoassay (CLIA) methods for measuring FT4 do not require segregation of the free fraction of T4, and therefore are less labor intensive and require less specialized technical training compared to ED. As such, veterinary diagnostic laboratories might be tempted to switch from ED to CLIA.

However, we and others occasionally have noted discordant results in FT4 concentrations determined by CLIA and ED in TGA-positive hypothyroid dogs.[b]

The purpose of this study was to compare concentrations of FT4 measured by semi-automated CLIA to subnormal concentrations of FT4 determined by ED in hypothyroid dogs that tested positive for TGA.

Materials and Methods

Dogs

The electronic database of the Animal Health Diagnostic Center of the College of Veterinary Medicine at Cornell University and the assay ledgers of the New York State Diagnostic Endocrinology Laboratory were searched for dogs that tested positive for TGA from March 1, 2014 through August 31, 2014. During this 6-month period, 35 dogs tested positive for TGA. Fifteen of the 35 TGA-positive dogs had sufficient serum stored in plastic tubes and frozen at -20°C to complete comprehensive thyroid profiles (FT4 by ED and CLIA, T4, T3, and TSH). These samples were neither grossly lipemic nor hemolyzed. Hypothyroidism was ultimately diagnosed in 13 of the 15 TGA-positive dogs based on concentrations of FT4 by ED below the reference range and other supportive tests of thyroid function.

The 13 TGA-positive hypothyroid dogs represented 7 breeds (Golden Retriever [n = 4], mixed breed [n = 2], Boxer [n = 2], and 1 each Coonhound, Doberman pinscher, Labrador retriever, Rhodesian ridgeback, and breed not characterized). Seven (54%) dogs were female (2 intact and 5 spayed) and 6 (46%) were male (2 intact and 4 castrated). Age ranged from 2.5 to 13 years (median, 6.5 years) for the 11 dogs with available age information.

Thyroid tests

Canine TGA had been qualitatively assessed as positive, negative, or inconclusive by an enzyme-linked immunosorbent assay properly blanked for nonspecific binding.[3,c] Total T4 and T3 concentrations were measured by commercially available RIA kits validated for use in dogs.[6–8,d,e] Free T4 concentration was determined by a commercially available kit validated for use in dogs that incorporated ED with RIA.[6,f] All T4, T3, and FT4 ED assays were run in duplicate with the results averaged for the final reported concentration. Free T4 concentration also was measured by use of human and veterinary FT4 semi-automated CLIA (FT4h CLIA and FT4v CLIA, respectively).[g,h] Both CLIAs are solid phase, enzyme-linked chemiluminescent competitive immunoassays with the solid phase being a bead coated with monoclonal murine anti-T4 antibody. The human and veterinary FT4 CLIAs differ in their adjustors, wherein T4 is lyophilized in either processed human or canine serum, respectively.[i,j] The CLIA analytical sensitivities were 0.07 ng/dL for FT4v and 0.13 ng/dL for FT4h.[i,j] The detection limits of the assays were: T4 0.05 μg/dL, FT4 ED 0.15 ng/dL, FT4h CLIA 0.13 ng/dL, and FT4v CLIA 0.13 ng/dL. Measurements less than these concentrations were assigned these values for statistical purposes. Concentration of TSH was determined using a canine TSH semi-automated CLIA.[9,k]

Discontinuation of the FT4 2-step RIA[l] necessitated a change in the non-ED method for measuring FT4 in dogs. In the New York State Veterinary Diagnostic Endocrinology Laboratory, we compared FT4 concentrations in canine serum samples (n = 109) analyzed by FT4 2-step RIA, FT4v CLIA, and FT4h CLIA. The canine internal quality control samples that we routinely analyze in the FT4 ED assay also were analyzed in each run of the FT4 2-step RIA, FT4v CLIA, and FT4h CLIA. Both CLIAs met all of the quality criteria that were established for the FT4 2-step RIA.

In the cross-assay comparison of patient samples, we found that concentrations of FT4h CLIA (range, 0.04–3.83 ng/dL) correlated better ($r = 0.85$, $P < .001$) with FT4 2-step RIA concentrations (range, 0.06–3.97 ng/dL) than did FT4v CLIA (range, 0.01–8.24 ng/dL; $r = 0.82$; $P < .001$). Therefore, upon discontinuation of the FT4 2-step RIA, our laboratory offered the FT4h CLIA as a non-ED option for measuring FT4 in dogs. For our study, this explains why the FT4h CLIA was given priority over the FT4v CLIA in the analysis of TGA-positive dogs. As a result, some dogs had insufficient serum sample to be analyzed by the FT4v CLIA.

At the time of sample submission, our laboratory followed the submitting veterinarian's request for method of FT4 analysis (ED [n = 4], non-ED [n = 3 2-step RIA; n = 5 human CLIA], or none [n = 1]). Eleven of 13 samples frozen at -20°C after original analysis were thawed on the same date and batch-analyzed to complete missing data. The sample from dog 13 was analyzed upon submission for FT4 by both ED and CLIA without previous storage, the sample from dog 8 was analyzed for FT4 by both ED and CLIA after 1 month of frozen storage, and the sample from dog 1 had all FT4 determinations completed at an earlier date. Limited volume of some samples precluded repeat analysis of tests that had already been completed during original sample submission. During the batch analysis for FT4 measurements, 9 of 13 (70%) samples were tested by ED, 7 of 8 (88%) by veterinary CLIA, and 6 of 13 (46%) by human CLIA.

Statistical analysis

Dogs with FT4 concentrations below the reference range by ED or CLIA (FT4h and FT4v) were considered to have evidence of hypothyroidism. Using dogs with concentrations of FT4 by ED below the reference range as the "gold standard", the sensitivities and false-negative rates of FT4h and FT4v by CLIA for diagnosing hypothyroidism were determined using standard formulas for these statistics.[10] Ninety-five percent CI were calculated for these proportions using the Wilson Score method with continuity correction.[11] Assuming a null hypothesis of complete agreement (i.e., no false-negative results), a 1 proportion test was performed to test the hypothesis that the true false-negative rate was 0%. P values <.05 were considered statistically significant.

Results

Concentrations of FT4 ED, FT4h CLIA, FT4v CLIA, total T4, total T3, and TSH in the 13 TGA-positive dogs are displayed in Table S1. Each dog's comprehensive thyroid profile supported a diagnosis of hypothyroidism.[12] In addition to subnormal concentrations of FT4 by ED in all dogs, 12 of 13 (92%) dogs had subnormal concentrations of T4 and 11 of 13 (85%) had increased concentrations of TSH. Interestingly, T3 concentrations were within the reference range in 8 of 12 (67%) dogs, and only decreased in 2 of 12 (16.5%) dogs. Three of 13 TGA-positive hypothyroid dogs (dogs 1, 6, and 13) had either an increased T4 or T3 concentration.

Five of 13 (38%) dogs with subnormal FT4 concentrations by ED had normal (n = 4) to high (n = 1) concentrations of FT4h by CLIA. Two of 8 (25%) dogs with subnormal FT4 concentrations by ED had normal (n = 1) or high (n = 1) concentrations of FT4v by CLIA. The increased FT4h and FT4v concentrations by CLIA were identified in the same dog (dog 1; Table S1). The percentages of false-negative results for FT4

determined by CLIA were significantly ($P < .0001$ for FT4h and $P < .001$ for FT4v) higher than the hypothesized false-negative rate of 0%. For detecting hypothyroidism in these TGA-positive dogs, sensitivities of the CLIA for FT4h and FT4v were 62% (95% CI, 32–85%) and 75% (95% CI, 36–96%), respectively, compared to FT4 by ED.

For the 5 dogs with discordant FT4 ED and FT4h CLIA results, total T4 was increased in 1 dog and decreased in 4; total T3 was increased in 1, within the reference range in 2, and decreased in 1 (with 1 missing result); and, TSH was increased in 5. For the 2 dogs with conflicting FT4 ED and FT4v CLIA results, total T4 was increased in 1 and decreased in 1 dog, whereas total T3 was within the reference range and TSH was increased in both dogs.

Discussion

In this study population, 25–38% of TGA-positive hypothyroid dogs had FT4 concentrations measured by CLIA that did not support a diagnosis of hypothyroidism. These findings would have led to inappropriate clinical decisions if interpreted without the benefit of more extensive thyroid testing. Therefore, caution should be exercised when FT4 measured by CLIA alone is used to screen dogs for hypothyroidism. In dogs suspected of hypothyroidism that test positively for TGA, normal or increased FT4 concentrations by CLIA may not exclude a diagnosis of hypothyroidism.

It remains unclear why a subset of TGA-positive hypothyroid dogs had disparate results for FT4 measured with CLIA compared to ED. In dogs the gold standard for FT4 determination is ED, a technique allowing separation of bound from FT4, followed by a sensitive RIA to measure FT4 in the dialysate.[2] With the CLIA method, FT4 in the patient's sample competes with enzyme-conjugated T4 for a limited number of binding sites on a bead coated with monoclonal murine anti-T4 antibody.[i,j] After washing, chemiluminescent substrate is added and activated by the bound enzyme, generating a signal inversely proportional to the patient's FT4 concentration.[i,j] Although the manufacturer has incorporated several features in the CLIA to accurately measure FT4, circulating antibodies to T4, hormone binding inhibitors, and heterophilic antibodies may cause interference in humans.[13,14,i]

Interestingly, despite subnormal concentrations of FT4 by ED, dog 1 (Table S1) had increased FT4h and FT4v concentrations by CLIA as well as increased total T4 concentration. Circulating T4 antibodies may spuriously increase total T4 concentrations when single-step RIA systems with antibody-coated tubes, such as the system used here, are employed.[1,2,15] In these assays, the radioactive tracer competes with endogenous T4 for the anti-T4 antibodies affixed to the tube. Once the tube is emptied, the remaining radioactivity in the tube is inversely proportional to the T4 concentration in the patient's sample. When circulating T4 antibodies are present, they can bind tracer resulting in a decreased radioactive tube count that would be misinterpreted as

an increased T4 concentration. Thyroid hormone antibody determinations were not performed in this study, but it is possible that circulating canine T4 antibodies also may bind enzyme-conjugated T4 in the CLIA causing a decreased signal that would be misconstrued as an increased FT4 concentration.

The other dogs (dogs 5, 7, 8, 13; Table S1) with discordant FT4 results did not have T4 concentrations that would suggest the presence of circulating T4 antibodies. Instead, dogs 5 and 8 had total T4 concentrations below the limit of assay detection (0.05 µg/dL), and dogs 7 and 13 had measurable total T4 concentrations that were less than the lower limit of the reference range. For dog 7, the FT4 concentration by ED was only slightly below the lower limit of the reference range, but the diagnosis of hypothyroidism was supported by the simultaneously increased TSH concentration. It remains possible that T4 antibodies were present in concentrations sufficient to interfere with CLIA measurement of FT4, but inadequate to affect RIA determination of T4.

The role of circulating T3 antibodies in the genesis of discordant FT4 results between ED and CLIA methods is uncertain. Using a single-step RIA system with antibody-coated tubes as employed in our study, circulating T3 antibodies would be expected to falsely increase total T3 concentrations.[1] For the 5 dogs with disparate FT4 ED and CLIA results, T3 was decreased in 1 dog (20%), within the reference range in 2 dogs (40%), increased in 1 dog (20%), and missing in 1 dog (20%). Although the dog with increased T3 concentration (dog 13, 1.63 ng/mL; reference range, 0.6–1.4 ng/mL) might have had circulating T3 antibodies, another dog with increased T3 concentration (dog 6, 2.91 ng/mL) had no difference between FT4 ED and FT4h CLIA concentrations (Table S1). Moreover, for the 12 dogs that had T3 measured, concentrations were decreased in 2 (16.5%), within the reference range in 8 (67%), and increased in 2 (16.5%) dogs. The high percentage of hypothyroid dogs with T3 concentrations within the reference range might be attributable to interference in test results from circulating T3 antibodies, but also may reflect the limited usefulness of T3 as a diagnostic test for hypothyroidism in the dog.[1,12] Previous studies have shown substantial overlap in ranges of T3 concentrations among healthy dogs, dogs with hypothyroidism, and dogs with nonthyroidal illness.[1,12,16,17]

Our study has limitations inherent in any retrospective evaluation. As a diagnostic laboratory, we followed the submitting veterinarian's request for thyroid testing at the time of sample submission. Subsequently, we batch-analyzed samples to complete missing data. Insufficient sample volume sometimes precluded repeat analysis of the data generated during the original submission. We tried to minimize the influence of different assay runs on results by storing samples frozen at $-20°C$ until a single thaw for batch analysis, using the same lot numbers for FT4 CLIA methods, and consistently incorporating quality control samples in each assay run. The laboratory's quality control sample (an aliquoted pool of canine serum) also was stored frozen

at $-20°C$, and its FT4 concentration remained stable throughout the study period under all assay conditions. Overall, we believe that sample handling and storage had negligible effects on the discordant results between FT4 ED and CLIA in the 5 dogs (1, 5, 7, 8, and 13) of our study. The fact that 2 of these dogs (8 and 13) had their ED and CLIA results analyzed at the same time (either directly at submission [dog 13] or after 1 month of frozen storage [dog 8]) supports our hypothesis that storage conditions do not explain the discordant results between the ED and CLIA methods. In addition, the 3 remaining dogs (1, 5, and 7) with disparate FT4 results originally were tested by ED, but then later tested by CLIA on samples stored according to the CLIA manufacturer's recommendations $(-20°C)$ for 2 months or less. [i,j] Nevertheless, future prospective studies completing comprehensive thyroid assessments concurrently during sample submissions from TGA-positive hypothyroid dogs would provide additional useful information.

In conclusion, caution should be exercised when FT4 measured by CLIA alone is used to screen dogs for hypothyroidism. In our study population, 25–38% of TGA-positive hypothyroid dogs had FT4 results determined by CLIA that did not support the diagnosis of hypothyroidism. Seemingly, FT4v CLIA produced fewer discordant results than FT4h CLIA when compared to FT4 ED, but verification of these findings in a larger case series of TGA-positive hypothyroid dogs is indicated. The CI for both the sensitivity and false-negative rates are broad, and whether FT4h CLIA and FT4v CLIA differ in the proportion of false test results is not possible to determine with a high degree of confidence given the small number of dogs studied. Using a conservative estimate that one-third of hypothyroid dogs are TGA-positive, the findings of this study would suggest that approximately 10% of all hypothyroid dogs might have misleading FT4 concentrations when measured by semi-automated CLIA. In these cases, more comprehensive thyroid evaluation (FT4 ED, T4, TSH, TGA) may help avoid diagnostic errors.

Footnotes

[a] IDEXX Laboratories, Inc. Current concepts on diagnosing and managing thyroid disease in dogs and cats. Advertorial, NAVC Clinician's Brief. Nov 2010;1–12.

[b] Peterson ME. Treatment and monitoring of hypothyroid dogs with thyroid hormone antibodies. Insights into Veterinary Endocrinology. pp. 4–7. Posted May 29, 2014. http://endocrine-vet.blogspot.com/search/label/Thyroid.

[c] Canine Thyroglobulin Autoantibody Immunoassay, Oxford Laboratories, Oxford, MI 48371.

[d] Coat-A-Count® Total T4, Siemens Healthcare Diagnostics, Tarrytown, NY 10591-5097.

[e] Coat-A-Count® Total T3, Siemens Healthcare Diagnostics, Tarrytown, NY 10591-5097.

[f] Free T4 – by Equilibrium Dialysis, ANTECH Diagnostics, Irvine, CA 92614.

[g] IMMULITE®/IMMULITE 1000 Free T4, Siemens Healthcare Diagnostics, Tarrytown, NY 10591-5097.

[h] IMMULITE®/IMMULITE 1000 Veterinary Free T4, Siemens Healthcare Diagnostics, Tarrytown, NY 10591-5097.

[i] IMMULITE®/IMMULITE 1000 Free T4 Package Insert, 2013:1–31; Siemens Healthcare Diagnostics, Tarrytown, NY 10591–5097.

[j] IMMULITE®/IMMULITE 1000 Veterinary Free T4 Package Insert, 2010:1–7; Siemens Healthcare Diagnostics, Tarrytown, NY 10591–5097.

[k] IMMULITE®/IMMULITE 1000 Canine TSH, Siemens Healthcare Diagnostics, Tarrytown, NY 10591-5097.

[l] GammaCoat® Free T4 (Two-Step) ^{125}I RIA Kit, DiaSorin, Stillwater, MN 55082–0285.

References

1. Feldman EC, Nelson RW, eds. Canine and Feline Endocrinology and Reproduction, 3rd ed. St. Louis, MO: Saunders, 2004;85–151.

2. Ferguson DC. Testing for Hypothyroidism in Dogs. Vet Clin Small Anim 2007;37:647–669.

3. Nachreiner RF, Refsal KR, Graham PA, et al. Prevalence of autoantibodies to thyroglobulin in dogs with nonthyroidal illness. Am J Vet Res 1998;59:951–955.

4. Dixon RM, Mooney CT. Canine serum thyroglobulin autoantibodies in health, hypothyroidism, and non-thyroidal illness. Res Vet Sci 1999;66:243–246.

5. Lee J-Y, Uzuka Y, Tanabe S, et al. Prevalence of thyroglobulin autoantibodies detected by enzyme-linked immunosorbent assay of canine serum in hypothyroid, obese, and healthy dogs in Japan. Res Vet Sci 2004;76:129–132.

6. Behrend EN, Kemppainen RJ, Young DW. Effect of storage conditions on cortisol, total thyroxine, and free thyroxine concentrations in serum and plasma of dogs. J Am Vet Med Assoc 1998;212:1564–1568.

7. Reimers TJ, McCann JP, Cowan RG, et al. Effects of storage, hemolysis, and freezing and thawing on concentrations of thyroxine, cortisol, and insulin in blood samples. Proc Soc Exp Biol Med 1982;170:509–516.

8. Reimers TJ, Lamb SV, Bartlett SA, et al. Effects of hemolysis and storage on quantification of hormones in the blood samples in dogs, cattle, and horses. Am J Vet Res 1991;52:1075–1080.

9. Marca MC, Loste A, Orden I, et al. Evaluation of canine serum thyrotropin (TSH) concentration: comparison of three analytical procedures. J Vet Diagn Invest 2001;13:106–110.

10. Dawson B, Trapp RG. Basic and Clinical Biostatistics. 4th ed. New York: McGraw-Hill; 2004:306–307.

11. Newcombe RG. Two-sided confidence intervals for the single proportion: comparison of seven methods. Statist Med 1998;17:857–872.

12. Peterson ME, Melian C, Nichols R. Measurement of serum total thyroxine, triiodothyronine, free thyroxine, and thyrotropin concentrations for the diagnosis of hypothyroidism in dogs. J Am Vet Med Assoc 1997;211:1396–1402.

13. Boscato LM, Stuart MC. Heterophilic antibodies: a problem for all immunoassays. Clin Chem 1988;34:27–33.

14. Beaman J, Woodhead JS, Liewendahl K, et al. The evaluation of a chemiluminescent assay for free thyroxine by comparison with equilibrium dialysis in clinical samples. Clin Chim Acta 1989;186:83–90.

15. Kemppainen RJ, Young DW, Behrend EN, et al. Autoantibodies to triiodothyronine and thyroxine in a golden retriever. J Am Anim Hosp Assoc 1996;32:195–198.

16. Nelson RW, Ihle SL, Feldman EC, et al. Serum free thyroxine concentration in healthy dogs, dogs with hypothyroidism, and euthyroid dogs with concurrent illness. J Am Vet Med Assoc 1991;198:1401–1407.

17. Miller AB, Nelson RW, Scott-Moncrieff JC, et al. Serial thyroid hormone concentrations in healthy euthyroid dogs, dogs with hypothyroidism, and euthyroid dogs with atopic dermatitis. Br Vet J 1992;148:451–458.

Supporting Information

Additional Supporting Information may be found online in Supporting Information:

Table S1. Thyroid profiles on 13 dogs with hypothyroidism that tested positive for thyroglobulin antibody (TGA) and had free thyroxine (FT4) concentrations below the reference range as determined by equilibrium dialysis (ED)