Measurement of total thyroxine concentration in serum from dogs and cats by use of various methods

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Objective—To compare results obtained from assay of total thyroxine (T₄) concentration in serum of dogs and cats by use of 4 methods.

Sample Population—Serum samples obtained from 98 dogs and 100 cats and submitted by veterinarians to an endocrine testing laboratory.

Procedure—Total T₄ concentration was determined in each sample by use of 4 assay methods. Assay methods included a radioimmunoassay (RIA) marketed for use in dogs, an RIA for use in humans, a chemiluminescent enzyme immunoassay for use in humans, and an in-house ELISA.

Results—Total T₄ concentrations obtained by use of all methods were significantly correlated. Bias-plot comparison revealed similar good overall agreement. Total T₄ concentrations determined by use of the RIA marketed for use in dogs were generally lower than concentrations measured by use of the other methods. Clinical comparisons were made by evaluation of the T₄ results in the context of the reference range recommended by each laboratory. A difference was found for clinical comparisons on the basis of T₄ assay method when used to identify dogs as possible hypothyroid suspects. This difference was related more to the reference range used than to the absolute T₄ value. The number of hypothyroid-suspect cats with T₄ values greater than the reference range was the same for each of the 4 assay methods.

Conclusions and Clinical Relevance—Total T₄ concentrations determined in dogs and cats by use of 4 commonly used methods provided similar and consistent results. (Am J Vet Res 2006;67:259–265)

Measurement of total T₄ concentrations in dogs and cats is commonplace in veterinary practice. In dogs, the test is most often used to exclude a diagnosis of hypothyroidism or to assess the adequacy of T₄ replacement treatment. In dogs suspected of being hypothyroid that have borderline concentrations within the reference range or low T₄ concentrations, additional tests, such as determination of free T₄ or thyrotropin (ie, thyroid-stimulating hormone) concentrations, are usually performed to confirm the diagnosis. In cats suspected of being hyperthyroid, the finding of an increased T₄ concentration is usually sufficient to confirm the diagnosis. Concentrations of T₄ in the mid- to upper portion of the reference range in hyperthyroid-suspect cats are considered suspicious or suggestive of the disease and warrant additional testing, such as measurement of free T₄ concentrations.

Increasingly, new methods and technologic advances for measurement of T₄ concentrations are being developed. The RIA method has been used for many years and may justifiably be considered a criterion-referenced standard. Results obtained by use of RIAs vary, probably as a result of differences in calibrators and their media, the antisera used, and the method used to separate bound T₄ from free T₄. Most RIA kit procedures are designed for use in samples obtained from humans but have been validated for use in samples obtained from domestic species. One total T₄ RIA kit is marketed as specific for use in canine samples. Technologic advances in immunoassays have allowed for methods that avoid the use of radioisotopes and, in addition, permit automation. For example, chemiluminescent- and ELISA-based systems have been validated for determination of total T₄ concentration in serum obtained from dogs and cats. Another ELISA for measurement of total T₄ concentration is commercially available for use by veterinarians in a practice setting.

Relatively few studies have reported total T₄ concentrations determined by use of various assay methods. In 1 study, investigators compared total T₄ concentrations measured in samples obtained from dogs, cats, and horses by use of RIA, chemiluminescent immunoassay, and CEIA. Analysis of results of that study revealed that although values obtained by use of RIA agreed well with results obtained by use of CEIA, total T₄ concentration determined by use of chemiluminescent immunoassay was 30% to 40% lower. In another study, total T₄ concentration in serum obtained from dogs and cats was determined by use of an RIA kit validated for use in dogs, and values were compared with those obtained when assayed by use of an in-house ELISA. Although T₄ concentrations determined by use of the 2 methods were moderately correlated, evaluation of bias plots and clinical assessment made on the basis of assay results revealed substantial disagreement. However, these findings differ from those for a preliminary study in which investigators compared total T₄ concentrations measured by use of RIA and CEIA.

<table>
<thead>
<tr>
<th>T₄</th>
<th>Thyroxine</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>CEIA</td>
<td>Chemiluminescent enzyme immunoassay</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
</tbody>
</table>

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sured by use of the RIA and CEIA methods with results obtained by use of an in-house ELISA. In that study, total \( T_4 \) concentrations measured by use of all 3 methods had excellent agreement. Reasons for conflicting results among the aforementioned studies are unclear.

The objective of the study reported here was to perform a comprehensive examination and comparison of total \( T_4 \) concentrations determined in single serum samples by use of 4 methods. Serum samples from dogs and cats submitted to a veterinary endocrine diagnostic laboratory were used. Data were compared by statistical tests and evaluation of clinical recommendations, and decisions were made on the basis of the results and reference ranges for each assay. Particular attention was given to comparing results obtained by use of an in-house ELISA with those obtained by use of the other methods.

**Materials and Methods**

**Sample population**—Serum samples obtained from 98 dogs and 100 cats and submitted to a veterinary endocrinology laboratory at our university for determination of \( T_4 \) concentration were used in the study. Samples selected for this study met 2 criteria. First, the \( T_4 \) concentration determined in the remaining 18 dogs was unknown. For cats, 64 samples were submitted to screen for hyperthyroidism, and an additional 17 were used to assess treatment adequacy of treatment in dogs receiving \( T_4 \) replacement treatment. The reason for the request to measure \( T_4 \) concentration in the remaining 18 dogs was unknown. For cats, 64 samples were submitted to screen for hyperthyroidism, and an additional 17 were used to assess treatment in cats receiving methimazole. The reason for performing the assay on the remaining 19 samples was not known. Most of the cats tested for hyperthyroidism had signs suggestive of the disease (typically weight loss in an older cat). Samples with pronounced lipemia or hemolysis were not used.

**\( T_4 \) assays**—The \( T_4 \) concentration in each sample was determined by use of 4 validated assays. Samples were initially assayed by use of a canine total \( T_4 \) coated-tube RIA (assay A). Subsequently, total \( T_4 \) was measured by use of an ELISA (ie, in-house ELISA; assay B) as described elsewhere; assay B was conducted by use of a diagnostic analyzer that is currently marketed for use by veterinarians. These 2 assays were performed at the university veterinary endocrinology diagnostic laboratory. The RIA was performed by laboratory technicians, whereas the in-house ELISA was performed by a veterinary student who had experience with the assay. The remaining aliquots were mailed to a company where they were assayed by use of an RIA total \( T_4 \) kit (assay C) and by use of a CEIA method (assay D), both marketed for use in humans and validated for use in dogs and cats.

Precision estimates were determined for each assay. Serum obtained from dogs and cats was serially diluted and assayed by use of each method; each method yielded displacement lines parallel to the line for the standard. Any \( T_4 \) result that initially exceeded the highest calibrator was serially diluted by use of the assay calibration diluent and reanalyzed.

Sensitivity was 0.15, 0.5, 0.25, and 0.4 µg/dL for assays A, B, C, and D, respectively. Sensitivity for assays A, C, and D was determined by calculating the concentration of \( T_4 \) that caused a significant \((P < 0.05)\) difference in percentage binding, compared with binding for the 0 µg/dL standard. Each assay was performed in accordance with the respective manufacturer's recommendations.

**Data analysis**—Least squares linear regression and calculation of the Pearson product moment correlation were used to compare overall agreement of \( T_4 \) values among assays. In addition, mean \( T_4 \) results were compared by use of a 1-way ANOVA, and the \( \chi^2 \) test was used to compare proportions. All analyses were performed by use of statistical analysis software and were conducted in accordance with established methods. Bias plots were constructed in accordance with the method of Bland and Altman by plotting the mean \( T_4 \) values obtained by use of 2 methods on the x-axis and plotting the difference between their mean \( T_4 \) values on the y-axis.

For categoric comparisons, canine \( T_4 \) values obtained by use of each assay were classified as low, clinically normal, or high on the basis of ranges recommended by the various laboratories (assays A, C, and D) or the kit manufacturer (assay B; Appendix). A similar comparison was made for samples obtained from cats. The laboratory ranges were originally established by assay of serum samples obtained from healthy dogs and cats. Additionally, \( T_4 \) values measured for the 41 dogs screened for hypothyroidism were examined to determine the number that would be considered hypothyroid suspects on the basis of detecting a \( T_4 \) concentration in the low range. Similarly, \( T_4 \) values measured for the 64 cats suspected of being hyperthyroid were examined to determine the number that would be categorized as consistent with the disease on the basis of detecting a \( T_4 \) concentration in the high range.

**Results**

The data allowed for a total of 12 pairs of comparisons for the 4 assay methods and values for 2 species.
Precision estimates were determined for each assay (Table 1). Regression analysis revealed that $T_4$ values obtained by use of each method were significantly correlated (Figures 1 and 2; Table 2). Analysis of bias plots indicated that results generally agreed well (bias value of approx 0) with $T_4$ values in the mid- to low range. Greater divergence was seen in a few samples containing higher $T_4$ concentrations.

Overall, $T_4$ values measured by use of assay A yielded lower $T_4$ concentrations in serum from both
species, which was reflected in the slopes of the regres-
sion lines (steeper slope) and in the bias plots. This dif-
ference was more profound as T₄ concentrations
increased and was more obvious in samples obtained
from cats. This pattern was further supported by the
finding that the mean T₄ concentration of all samples
obtained from dogs measured by use of assay A (mean
± SD, 3.3 ± 2.0 µg/dL) was significantly (P = 0.01)
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obtained from dogs measured by use of assay A (mean
± SD, 3.3 ± 2.0 µg/dL) was significantly (P = 0.01)

Figure 2—Linear regression graphs (A, C, and E) and bias plots (B, D, and F) of pairwise comparisons of serum total T₄ concentrations in cats measured by use of 4 assays. Only data comparing results for assay B with results for the other assays are shown, but they are representative of other comparisons among the 4 assays. See Figure 1 for remainder of key.
lower than that measured by use of assays B, C, or D (3.9 ± 2.7 µg/dL, 3.6 ± 2.4 µg/dL, and 3.5 ± 2.6 µg/dL, respectively). Similar results were found when mean concentrations were compared for serum samples obtained from cats. For an unknown reason, a bimodal distribution was apparent in serum T₄ values for dogs measured by use of assay B, compared with results obtained by use of assay D (Figure 1). This distribution accounted for a relatively lower correlation (Table 2).

Reference ranges recommended by the testing laboratories (assays A, C, and D) or the kit manufacturer (assay B) were used to categorize results; the number of concordant results was compared. The proportion of animals assigned to each category did not vary significantly among the various assay methods. To investigate the potential influence of source of T₄ result on clinical decisions, T₄ values in the 41 dogs screened for hypothyroidism were evaluated. Each T₄ concentration was compared with its respective reference range. The number of dogs with low T₄ values categorized as hypothyroid-suspect dogs (ie, value in the low range) or clinically normal dogs. None of the 41 dogs had a T₄ result in the high range. The number of dogs with low T₄ values categorized on the basis of results for assays A to D was 25, 14, 14, and 22, respectively; the number of dogs differed significantly among assays. Assays A and D used the same T₄ value to define the lower limit of the reference range (ie, 1.6 µg/dL), whereas assays B and C used another value (ie, 1.3 µg/dL). To examine the influence of the cutoff value on categorization, the 41 dogs were recategorized on the basis of a common T₄ cutoff value of 1.45 µg/dL, which was a value midway between 1.3 and 1.6 µg/dL. The number of dogs with a low T₄ concentration by use of this intermediate cutoff value was 20, 20, 18, and 19 for assays A to D, respectively; the number of dogs did not differ significantly among assays. Sixteen of these dogs had a total T₄ concentration in the low range when measured by use of all 4 assay methods.

A similar categorization was made by use of the data for serum samples obtained from 64 hyperthyroid-suspect cats. For this categorization, cats were classified as clearly consistent with hyperthyroidism (T₄ in the high range) or not consistent with hyperthyroidism (T₄ in the low or clinically normal range). The number of cats with a high T₄ concentration was similar when results for all assays were compared (29, 27, 30, and 30 cats for assays A to D, respectively). Twenty-five of these cats had a high T₄ concentration consistent with hyperthyroidism when measured by use of all 4 assays.

Discussion

The study reported here was conducted to achieve 2 main objectives. First, because of conflicting findings related to the accuracy and validity of the in-house ELISA method for total T₄ measurement, a more comprehensive evaluation of this assay was obtained by comparing serum T₄ concentrations measured in serum samples obtained from dogs and cats by use of the in-house ELISA and 3 other validated methods. The second objective was to assess the variation in total T₄ concentrations and the clinical assessment of these results, similar to the situation that veterinarians may encounter when submitting samples to a reference laboratory. Interpretation of results is influenced by the method used to make the determination as well as by the reference ranges recommended by the laboratory for use in clinical assessment. A weakness of the study was that the exact clinical status and diagnoses for each patient were not known. Strengths of the study were that samples originated from clinical cases and that sufficient sample volume allowed for measurement by multiple T₄ assays.

Statistical comparisons of T₄ results revealed, in general, good correlation between T₄ concentrations measured by use of the 4 methods. Analysis of regression lines, correlations, and, more importantly, bias plots indicated that agreement was generally excellent for T₄ concentrations in the low to middle portion of the clinically normal ranges. A few divergent results were seen when comparing results for all methods at higher T₄ concentrations. It is possible that factors such as lipemia, hemolysis, or the method of serum collection (eg, use of serum separator collection tubes) contributed to these differences. A major finding was that total T₄ concentrations obtained by use of assay A (canine RIA) were generally lower than those determined by use of the other methods. This difference increased progressively as the T₄ concentration increased and was more profound in samples obtained from cats, as indicated by the slopes of the regression lines (Table 2). Factors likely contributing to the lower T₄ concentrations for assay A include the fact that assay A used calibrators (one of canine origin) and a lower calibration range that differed from those used by the other assays. In addition, assay A had the lowest sensitivity of all 4 methods. Overall, analysis of these data indicated that users of assay A should anticipate lower absolute total T₄ concentrations, compared with values obtained by use of the other 3 methods.

Table 2—Results from linear regression analysis of pairwise comparisons to contrast all possible pairs of assays* used to measure T₄ concentrations in serum samples obtained from dogs and cats.

<table>
<thead>
<tr>
<th>Assay pair</th>
<th>Slope</th>
<th>Intercept</th>
<th>r²</th>
<th>Slope</th>
<th>Intercept</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>A vs B</td>
<td>1.23</td>
<td>-0.22</td>
<td>0.87</td>
<td>1.43</td>
<td>-0.08</td>
<td>0.95</td>
</tr>
<tr>
<td>C vs B</td>
<td>0.93</td>
<td>0.23</td>
<td>0.91</td>
<td>0.99</td>
<td>0.64</td>
<td>0.94</td>
</tr>
<tr>
<td>D vs B</td>
<td>1.09</td>
<td>0.66</td>
<td>0.64</td>
<td>0.82</td>
<td>0.58</td>
<td>0.91</td>
</tr>
<tr>
<td>A vs C</td>
<td>1.23</td>
<td>-0.26</td>
<td>0.95</td>
<td>1.38</td>
<td>-0.53</td>
<td>0.93</td>
</tr>
<tr>
<td>A vs D</td>
<td>1.2</td>
<td>-0.18</td>
<td>0.71</td>
<td>1.63</td>
<td>-0.42</td>
<td>0.81</td>
</tr>
<tr>
<td>C vs D</td>
<td>0.93</td>
<td>0.23</td>
<td>0.91</td>
<td>1.19</td>
<td>0.2</td>
<td>0.97</td>
</tr>
</tbody>
</table>

See Table 1 for remainder of key.

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Veterinarians commonly submit serum samples for total T₄ measurement to screen dogs for hypothyroidism. The T₄ result is compared with the laboratory's reference range to assist in determining the likelihood that the dog is hypothyroid. Generally, the test is considered to be more useful in ruling out the disease, rather than in confirming the diagnosis. Dogs with T₄ concentrations below the reference range may or may not be hypothyroid because a multitude of factors can cause low total T₄ concentrations in euthyroid dogs. Generally, additional tests of thyroid function (measurement of free T₃ or thyroid-stimulating hormone) are recommended to confirm or reject the diagnosis for dogs with low T₄ concentrations. The study reported here enabled direct examination of the impact of the origin of the T₄ result on this categorization process. Analysis of our results revealed that the source of the T₄ result affected whether a dog was categorized as a hypothyroid suspect. However, the reason for this difference related more to the limits of the reference range, rather than to significant variations in total T₄ concentrations. The finding that the absolute T₄ concentrations were generally similar for the 4 assays for samples from the 41 hypothyroid-suspect dogs may appear surprising, especially given that assay A typically yielded lower T₄ concentrations. However, examination of bias plots revealed that T₄ concentrations in dogs agreed quite well on the basis of comparison of results for assay A with results for the other assays when hormone concentrations were approximately ≤ 6 µg/dL. Because we did not know with certainty which of these dogs was truly hypothyroid, it was not possible to comment on the reference range that was most accurate. Changes in the reference range will impact test specificity and sensitivity and therefore affect the number of false-positive and false-negative results. Overall, analysis of the results reported here indicated that these 4 methods had good agreement when segregating dogs into the 2 chosen categories (hypothyroid-suspect dogs and clinically normal dogs). It should be mentioned that there was a difference in categorization for some dogs even when a common reference range was used for all assays.

Most cats with hyperthyroidism have total T₄ serum concentrations above the reference range. Examination of T₄ concentrations in the 64 hyperthyroid-suspect cats revealed good agreement when comparing results for assay methods to identify those cats with T₄ concentrations above the reference range. As expected from the bias-plot data for these cats, T₄ concentrations determined by use of assay A were lower, compared with values derived by use of the other assays (Figure 2). The reference range used for assay A reflected this difference. It should be mentioned that the finding of a T₄ concentration within the reference range in a hyperthyroid-suspect cat cannot be used to rule out the disease. Some hyperthyroid cats, particularly those with early or mild disease or those with concurrent nonthyroidal illness, may have a total T₄ concentration within the reference range.

Analysis of results of the study reported here indicated that serum total T₄ concentrations measured by use of the in-house ELISA method (assay B) were generally in agreement with results of T₄ concentrations measured by use of the other 3 methods. This finding differs from that in another comparative study but concurs with the results of a preliminary report. In the study in which there was poor correlation, a single-pair comparison was made by use of results for the canine total T₄ RIA and the in-house ELISA. The fact that the canine RIA assay yielded lower T₄ values than did the in-house ELISA could have contributed to some of the discrepancy; however, it is unlikely that this factor accounted for most of the differences. A contributing factor likely was related to differences in precision by use of the in-house method when comparing results for that study with results for the study reported here. Furthermore, interassay CV was 18% and 28% for pooled serum obtained from dogs and cats, respectively, in that study. In the study reported here in which we used pooled canine serum and pooled feline serum (data not shown) that contained a similar concentration of T₄ to that in the other study (approx 2.5 µg/dL.), the intra-assay and interassay CVs associated with the in-house method were both ≤ 8%. The reason for this substantial difference in precision between the 2 studies is unclear. The in-house ELISA measurements were obtained by use of 2 instruments.

Another factor contributing to the differences between the studies relates to the influence of reference ranges on clinical decisions. Even in the study reported here, clinical categorizations made on the basis of results for the assay with the lowest CV (assay B) may not be the same as those made on the basis of results for the other 3 assays. It should be noted that use of our in-house ELISA method allowed comparison of results for our study with those reported in the literature. Examination of T₄ concentrations in the 64 hyperthyroid-suspect cats revealed good agreement when comparing results for assay methods to identify those cats with T₄ concentrations above the reference range. As expected from the bias-plot data for these cats, T₄ concentrations determined by use of assay A were lower, compared with values derived by use of the other assays (Figure 2). The reference range used for assay A reflected this difference. It should be mentioned that the finding of a T₄ concentration within the reference range in a hyperthyroid-suspect cat cannot be used to rule out the disease. Some hyperthyroid cats, particularly those with early or mild disease or those with concurrent nonthyroidal illness, may have a total T₄ concentration within the reference range.

Overall, analysis of these results supports the conclusion that the in-house ELISA method provides reliable and consistent total T₄ concentrations for serum samples obtained from dogs and cats. As with all test systems, including those in private veterinary clinics and reference laboratories, it is advisable that users periodically measure T₄ concentrations in stored pooled serum of dogs and cats to confirm consistent performance for the assay.

The findings reported here may not apply to T₄ concentrations measured by use of other assays and methods. Each of the 4 assays in this study has been validated for use in companion animals. Importantly, sensitivity of all these assays was a T₄ concentration of ≤ 0.5 µg/dL., which is a necessity when evaluating the relatively low concentrations of T₄ circulating in the bloodstream of dogs and cats. Analysis of the data reported here indicated good overall agreement for serum T₄ concentrations in samples obtained from dogs and cats measured by use of 4 assay methods, including an in-house ELISA. Differences in clinical decisions when evaluating dogs for hyperthyroidism related more to the reference range used, rather than to inconsistencies in results for the 4 assays.

   b. SNAP T₄, IDEXX Laboratories, Westbrook, Me.
   c. Peterson ME, DeMarco CL, Sheldon KM. Total thyroxine testing: comparison of an in-house test kit with radioimmuno-


**Appendix**

Reference ranges recommended by the various laboratories (assays A, C, and D) or the manufacturer of the kit (assay B) for 4 assays* used to measure T₄ concentrations in serum samples obtained from dogs and cats.

<table>
<thead>
<tr>
<th>Range</th>
<th>Dogs</th>
<th></th>
<th></th>
<th>Cats</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Low (µg/dL)</td>
<td>&lt; 1.6</td>
<td>&lt; 1.3</td>
<td>&lt; 1.3</td>
<td>&lt; 1.6</td>
<td>&lt; 1.0</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td>Clinically normal (µg/dL)</td>
<td>1.6–4.3</td>
<td>1.3–4.0</td>
<td>1.3–4.0</td>
<td>1.6–5.0</td>
<td>1.0–3.9</td>
<td>1.0–5.0</td>
</tr>
<tr>
<td>High (µg/dL)</td>
<td>&gt; 4.3</td>
<td>&gt; 4.0</td>
<td>&gt; 4.0</td>
<td>&gt; 5.0</td>
<td>&gt; 3.9</td>
<td>&gt; 5.0</td>
</tr>
</tbody>
</table>

*The 4 assays were as follows: a total T₄ coated-tube RIA validated for use in dogs (assay A), an ELISA* (ie, in-house ELISA; assay B), an RIA total T₄ kit marketed for use in humans* (assay C), and a CEIA method marketed for use in humans* (assay D).