Hypothyroidism is one of the most common endocrine disorders in dogs [6]. Half of canine hypothyroid cases were estimated to result from lymphocytic thyroiditis [6, 12, 14]. The resultant loss of thyroid functions leads to insufficient production and secretion of thyroid hormones [6]. Thyroid hormone deficiency can cause a variety of clinical symptoms such as sluggishness, obesity, skin diseases, cold intolerance, hair loss, and reproductive problems [6, 26]. Canine lymphocytic thyroiditis is considered to be an immune-mediated disease with clinical and histological similarities to Hashimoto’s thyroiditis in man [3, 22]. Detection of autoantibody specific for thyroglobulin (Tg) is an indispensable laboratory subject for the diagnosis of Hashimoto’s thyroiditis in human medicine. The antibody specific for Tg has been also found in a high proportion of clinically diagnosed hypothyroid dogs [2, 14, 16, 17]. Recently the reliability of enzyme-linked immunosorbent assay (ELISA) techniques for Tg autoantibody (TgAA) detection in dogs was compared with that of thyroid biopsy, and was demonstrated as excellent diagnostic methods with high sensitivity and specificity [1, 17, 24]. However, several studies of the occurrence of TgAA in nonhypothyroid dogs were reported. In a recent study, 38% of dogs with hypothyroidism were positive for TgAA and among clinically healthy dogs and dogs with other internal diseases, 14% and 25% were positive for TgAA, respectively [8]. In the assay used by Haines et al. [16], the number of TgAA positive dogs in non-hypothyroid dogs was high, so that some of the positive dogs may have been false-positive. These “false-positive” results were explained by the fact that the epitope recognized by TgAA in dogs with hypothyroidism differs from that in normal dogs positive for TgAA [17]. Thus, our hypothesis holds that there is the pathogenic epitopes recognized by TgAA on cTg in hypothyroid dogs. Investigators have studied the antigenic properties of human Tg to locate the epitope recognized by TgAA and to determine amino acid sequence of the epitopes. Serum samples from patients of Hashimoto’s thyroiditis examined by Saboori et al. expressed similar Western immunoblot-binding pattern against Tg tryptic peptides [29]. Although Verschueren et al. reported that cTg had similar properties to other animals [33], the antigenic properties of cTg have not been well characterized yet.

The aim of the present study is to determine the dominant epitopes recognized by TgAA on cTg molecule by comparing with the recognition patterns of circulatory TgAA in hypothyroid dogs. The information of dominant epitopes recognized by TgAA in hypothyroid dogs would aid in the development of accurate diagnoses of hypothyroidism in dogs and in the differential diagnosis of hypothyroid, non-hypothyroid with skin diseases, and normal dogs.

MATERIALS AND METHODS

Purification of canine thyroglobulin: cTg was purified according to the methods described in the reports [17, 30, 33]. Blood samples were collected from two clinically normal, purebred beagle dogs. The serum free thyroxine (FT₄) levels and thyroid stimulating hormone (TSH) levels were determined by the commercial laboratory of clinical examination (IDEXX Laboratories, KK, Tokyo, Japan). The beagle dogs were euthanized by intravenous injection of overdosed sodium pentobarbital. Thyroid gland tissues were isolated and immediately stored in liquid nitrogen until
the purification. The caudal part of each left thyroid lobe was submitted for histological examination.

The serum FT₄ and TSH levels were normal levels. The serum collections were used as normal controls and stored at −80°C. The thyroid glands showed normal morphological structure of the follicles without inflammatory infiltration.

After thawing, the thyroid glands were placed in phosphate buffer (9.2 mM Na₂HPO₄, 1.3 mM NaH₂PO₄, 150 mM NaCl, pH 7.4) with protease inhibitor (0.05 m/l g tissue, Protease Inhibitor Cocktail: SIGMA Chemical, St. Louis, MO, U.S.A.), homogenized, and centrifuged three times at 20,000 × g for 30 min at 4°C. The supernatant was then filtered with 0.45 μm pore size membrane filter. Finally total thyroid proteins were purified by gel filtration on the Sepharose CL-4B column (2.5 × 75 cm, Amersham Pharmacia Biotech UK, Little Chalfont, Buckinghamshire, UK) in phosphate buffer at flow rate of 1 ml/min. Fractions of 5 ml each were collected and stored at −80°C. Protein concentrations of the fractions were measured by the method of Bradford [4] using BSA (Albumin Bovine: SIGMA Chemical, St. Louis, MO, U.S.A.) as the standard.

Characterization of eluate fractions: The fraction samples, which contained more than 0.8% iodine [33] quantitated by the modified ammonium persulfate digestion on microplate (APDM) method [25], were evaluated on a Native-PAGE according to the method of Verschuuren et al. [33]. Five μg protein of each fraction was separated on the polyacrylamide gradient gel (4–20%, READY GELS: Bio-Rad Laboratories, Hercules, California, U.S.A.) and stained by diffusion with Coomassie Blue G-250 (Bio-Safe CBB G-250: Bio-Rad Laboratories, Hercules, California, U.S.A.). The position of the bands was compared with a mixture of protein standards (HMW Calibration Kit for Native electrophoresis: Amersham Pharmacia Biotech UK, Little Chalfont, Buckinghamshire, UK) containing thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and albumin (67 kDa) (Fig. 1).

cTg separated by Native-PAGE was transferred into PVDF paper (Immobilon-P: Millipore Corporation, Bedford, MA, U.S.A.). After transfer, the PVDF paper was treated by the rapid immunodetection method. The transferred membranes were washed twice with water, and dried, cut into multiple strips. The strips were exposed to mouse anti-human Tg monoclonal antibody (Thyroglobulin Ab-1, Clone 2H11: NeoMarkers, Fremont, CA, U.S.A.), which could be cross-reacted with cTg, diluted at 1:500 with 1% BSA in PBS (8.1 mM Na₂HPO₄, 137 mM NaCl, 2.68 mM KCl, 1.47 mM KH₂PO₄, pH 7.4) containing 0.05% Tween-20 for 1 hr at room temperature. After washing with PBS containing 0.05% Tween-20, the strips were treated with horseradish peroxidase-conjugated rabbit anti-mouse IgG (SIGMA Chemical, St. Louis, MO, U.S.A.) diluted at 1:2000 with 1% BSA in PBS containing 0.05% Tween-20 for 30 min at room temperature. Immunostaining of the strips was carried out using the kit (Konica immunostaining HRP-1000: Konica, Tokyo, Japan) according to the manufacturer’s protocol.

After Native-PAGE, an electrophoretic single band was identified with the purified porcine Tg marker (molecular weight: 669 kDa) (Fig. 1). The main peak fractions (Nos. 40 and 41) were used as the standards for ELISA and as trypsin digestion.

Animals and sample collection: Fifteen dogs, clinically suspected hypothyroidism and 4 normal dogs were studied. Blood samples were collected from cephalic vein before any therapeutic or diagnostic procedures. Serum samples were taken by centrifugation 1,700 × g for 30 min at 4°C and stored at −20°C until analyses. The serum FT₄ levels and TSH levels were determined by the commercial laboratory of clinical examination (IDEXX Laboratories, KK, Tokyo, Japan). Diagnosis of hypothyroidism was confirmed on the basis of clinical signs such as alopecia, lethargy, and weight gain as well as the following laboratory findings: hypercholesterolemia, low FT₄ levels and high TSH levels. Hypothyroidism was excluded by medical history, physical examination data, laboratory examination results, and responses to treatments. One of the dogs was not tested for laboratory examination, because of no owner’s compliance. In this case the definitive diagnosis of non-hypothyroid skin disease was based on improvement of dermatological condition by medical treatments for atopy and pyoderma. Four dogs that were examined in the purpose of the medical examination were defined as clinically normal by physical and laboratory data. Because of no owner’s compliance, these dogs were tested for either serum FT₄ levels or serum TSH levels. The dogs were classified to 2 groups: Seven dogs with hypothyroidism on the basis of hormonal abnormality of low FT₄ and high TSH levels: Eight dogs with dermatological diseases without hormonal abnormality of FT₄ and TSH. The present study was approved by the NIH guidelines, the regulations of the Local Institutional Animal Care and Use Committee and owners signed a consent form before enrolling their dogs.

ELISA for thyroglobulin autoantibody: Canine TgAA in the serum samples were detected by the modified method of Iversen et al. [17]. Microtiter plates (Falcon Micro Test III: Becton Dickinson Labware, Franklin Lakes, NJ, U.S.A.)
were coated with canine Tg diluted in 10 µg/ml of PBS and carbonate buffer (321 mM Na₂CO₃, 667 mM NaHCO₃, pH 9.6). After incubation for overnight at 4°C, plates were washed with PBS containing 0.05% Tween-20 and blocked with 5% skim milk in PBS. After washing, 50 µl of serum samples which were titrated in 2 fold dilutions beginning at 1/20 to 1/2,560 with 1% skim milk in PBS in duplicates were incubated, then the plates were washed again and incubated with horseradish peroxidase-conjugated sheep anti-dog IgG. After washing, O-phenylenediamine dissolved in citrate phosphate buffer (24 mM Citric acid, 51 mM Na₂HPO₄, pH 5.0) was added to each well and incubated at room temperature for 20 min. The enzyme-mediated reaction was stopped by 1 M H₂SO₄. The optical density (OD) was read at 490 nm wave length.

One of the TgAA positive sera was pooled as a calibrator. Each of OD values was calibrated with the mean values of calibrator (n=3 per plate) in each plate. A standard curve of negative for TgAA was constructed from duplicate tests of pooled sera from two clinically normal dogs that were euthanized for purification of thyroglobulin. Sample sera were judged to be positive at dilutions in which the OD values exceeded twice the SD above the negative curve. The intra-assay CV in the present assay was less than 5%. Each sample was assayed in duplicate in the same assay.

Trypsin digestion of canine thyroglobulin: Canine Tg was processed by the method of Saboori et al. [29]. In a preliminary experiment, cTg was digested at 37°C with TPCK-pretreated trypsin (10,000–15,000 BAEE units/mg: SIGMA Chemical, St. Louis, MO, U.S.A.) for different incubation times to determine the optimum conditions for trypsin degradation of Tg. The trypsin: cTg ratio at 1:10 (w/w) was optimal. Canine Tg was incubated with trypsin for 1 hr at 37°C. The reaction was stopped by SDS-PAGE sample buffer without the reducing agent, then incubated for 1 hr at 37°C.

SDS-PAGE and Western blot of tryptic fragments: SDS-PAGE was performed on a 4–20% gradient gel (4–20%, READY GELS J: Bio-Rad Laboratories, Hercules, California, U.S.A.) according to the method of Laemmli [19]. Approximately 10 µg of cTg tryptic fragments was separated and transferred into PVDF membrane (Immobilon-P: Millipore Corporation, Bedford, MA, U.S.A.). After transfer, the PVDF membrane was treated by the rapid immunodetection method. The transferred membranes were washed twice with water, and dried, cut into strips. The strips were exposed to sera diluted at 1:10 with 1% BSA in PBS with 0.05% Tween-20 for 1 hr at room temperature. After washing with PBS, the strips were treated with biotin-conjugated mouse monoclonal anti-dog IgG (SIGMA Chemical, St. Louis, MO, U.S.A.) diluted at 1:2,000 with 1% BSA in PBS containing 0.05% Tween-20 for 30 min at room temperature. The strips were washed and exposed to avidin-peroxidase conjugate diluted at 1:1,000 with 1% BSA in PBS containing 0.05% Tween-20 for 30 min at room temperature. After washing again, immunostaining of the strips was carried out using the kit of 3,3′-diaminobenzidine tetrahydrochloride (FAST DAB: SIGMA Chemical, St. Louis, MO, U.S.A.). The molecular weights of Tg tryptic peptides under non-reducing conditions of SDS-PAGE are expressed as an approximate molecular weight.

The recognition specificity of cTg tryptic fragments was examined by preincubating serum samples from TgAA-positive dogs added with purified cTg at the concentration of 0.5–0.75 mg/ml for 1 hr at 37°C.

Statistical analyses: Statistical analyses were conducted using the Stat View® program (SAS Institute Inc., Cary, NC, U.S.A.). Comparison of TgAA positive rate between the groups was submitted to Fisher’s exact probability test.

RESULTS

Purification of cTg: The Native-PAGE of elution fractions by the cTg chromatography on the Sepharose CL-4B was shown in Fig. 1. The peak fractions contained mainly Tg with a molecular weight of 669 kDa. The fractions 36 to 44 contained higher molecular weight Tg, and fractions 37 to 44 contained small but increasing amount of lower molecular weight Tg. The fractions corresponding to the descending part of the curve (fractions 42 to 45) contained approximate 140 kDa peptide. For each fraction, two major bands, one at 669 kDa and the other at lower molecular weight Tg, were detected by the Western blot using mouse anti-human Tg monoclonal antibody (Fig. 2).

Fig. 2. Western immunoblot patterns of elution fractions by the cTg chromatography on Sepharose CL-4B in comparison with human Tg (hTg) and standard protein (M). Each fraction was isolated by Native-PAGE and used in Western immunoblot. Fraction 36 is the ascending slope of the curve, fractions 40 and 41 are the peak fractions.
Autoantibody detection: Table 1 presents serum FT₄, TSH levels and TgAA titers in 7 hypothyroid dogs, 8 non-hypothyroid dogs with skin diseases, and 4 clinically normal dogs. Five of 7 hypothyroid dogs were positive for antibody against canine thyroglobulin. TgAA was also detected in 1 of 4 normal dogs and 1 of 8 dogs with skin diseases. Hypothyroid dogs had significantly (P<0.05) greater prevalence of TgAA than dogs with skin diseases. Of 5 hypothyroid TgAA-positive dogs, 4 were Golden Retrievers, and 3 of them showed high titers of TgAA.

Immunoreactivities of sera from hypothyroid dogs to Tg tryptic peptides: Tg proteolysis occurred rapidly, and the majority of fragments appeared after 1 hr of incubation. In the same tryptic condition, human Tg used as the positive control in this study appeared further degradation compared with canine Tg. The degradation patterns in human and canine Tg were significantly different each other (data not shown).

Results of various patterns produced by sera from TgAA-positive dogs are shown in Fig. 4. Serum samples from 3 dogs with high TgAA titers reacted broadly with high molecular weight more than 60 kDa (Lane 1, 2, and 5 in Fig. 4). The variety of immunoblot patterns was recognized in the dogs with high TgAA titers ranging from 45 to 90 kDa. These immunoblot patterns were disappeared after the pretreatment with sufficient amount of intact cTg (Fig. 5). All serum samples of both TgAA-positive dogs and negative controls reacted to low molecular weight ranging from 15 to 20 kDa. Peptide with 40 kDa was recognized in the sera from dogs with low TgAA-titer (Lane 4, 6, and 7 in Fig. 4). These immunoblot patterns of the sera were recognized after pretreatment with sufficient amount of intact cTg.

DISCUSSION

Canine Tg has the same chromatographical characters as Tg isolated from other species, including pig, sheep, goat, bovine, rat and man [10, 32]. The identical electrophoretical mobility of the elution fractions of the cTg chromatography in Native-PAGE has been shown [33]. The higher and lower molecular weight of Tg has previously been described as cTg dimer (1,300 kDa) and its subunit (330 kDa) [33]. In our study, the serum samples from TgAA-positive dogs reacted to several tryptic peptides with various patterns. The samples with high titer of TgAA reacted broadly to high molecular peptides. These characteristic immunob-

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**Table 1. Thyroglobulin autoantibody detected in hypothyroid dogs, non-hypothyroid dogs with skin diseases, clinically normal dogs**

<table>
<thead>
<tr>
<th></th>
<th>Female/Male</th>
<th>Age (year)</th>
<th>FT₄ (pmol/l)</th>
<th>TSH (ng/ml)</th>
<th>TgAA positive dogs</th>
<th>TgAA titer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Normal dogs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kisyu</td>
<td>F</td>
<td>3</td>
<td>19.5±3.6⁽A⁾</td>
<td>0.1⁽B⁾</td>
<td>1/4⁽B⁾</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Mixbreed</td>
<td>F</td>
<td>9</td>
<td>17.3</td>
<td>NT</td>
<td></td>
<td>320</td>
</tr>
<tr>
<td>Mixbreed</td>
<td>F</td>
<td>9</td>
<td>14.6</td>
<td>NT</td>
<td></td>
<td>&lt;20</td>
</tr>
<tr>
<td>English Pointer</td>
<td>M</td>
<td>12</td>
<td>26.6</td>
<td>NT</td>
<td></td>
<td>&lt;20</td>
</tr>
<tr>
<td><strong>Dogs with skin disease</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Golden Retriever</td>
<td>F</td>
<td>6</td>
<td>11.9</td>
<td>0.02</td>
<td></td>
<td>&lt;20</td>
</tr>
<tr>
<td>Shih Tzu</td>
<td>F</td>
<td>6</td>
<td>24.3</td>
<td>0.10</td>
<td></td>
<td>&lt;20</td>
</tr>
<tr>
<td>West Highland White Terrier</td>
<td>M</td>
<td>11</td>
<td>19.6</td>
<td>0.18</td>
<td></td>
<td>&lt;20</td>
</tr>
<tr>
<td>Shiba</td>
<td>F</td>
<td>6</td>
<td>NT</td>
<td>NT</td>
<td></td>
<td>&lt;20</td>
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<tr>
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<td>13.7</td>
<td>0.04</td>
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<td>&lt;20</td>
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<tr>
<td>Labrador Retriever</td>
<td>M</td>
<td>6</td>
<td>14.9</td>
<td>0.08</td>
<td>80</td>
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</tr>
<tr>
<td>Toy Poodle</td>
<td>M</td>
<td>1</td>
<td>11.5</td>
<td>0.25</td>
<td></td>
<td>&lt;20</td>
</tr>
<tr>
<td>Labrador Retriever</td>
<td>F</td>
<td>4</td>
<td>10.0</td>
<td>0.02</td>
<td></td>
<td>&lt;20</td>
</tr>
<tr>
<td><strong>Dogs with primary hypothyroidism</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Golden Retriever</td>
<td>F</td>
<td>8</td>
<td>3.0</td>
<td>1.10</td>
<td></td>
<td>&lt;20</td>
</tr>
<tr>
<td>Golden Retriever</td>
<td>F</td>
<td>6</td>
<td>3.0</td>
<td>2.20</td>
<td></td>
<td>&gt;2560</td>
</tr>
<tr>
<td>Golden Retriever</td>
<td>Cast</td>
<td>6</td>
<td>3.0</td>
<td>1.00</td>
<td></td>
<td>&gt;2560</td>
</tr>
<tr>
<td>Golden Retriever</td>
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<td>5</td>
<td>3.0</td>
<td>1.20</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Akita</td>
<td>Ovx</td>
<td>5</td>
<td>3.7</td>
<td>0.54</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Golden Retriever</td>
<td>M</td>
<td>2</td>
<td>3.0</td>
<td>0.54</td>
<td></td>
<td>&gt;2560</td>
</tr>
<tr>
<td>Shih Tzu</td>
<td>M</td>
<td>12</td>
<td>3.0</td>
<td>1.00</td>
<td></td>
<td>&lt;20</td>
</tr>
</tbody>
</table>

A) serum samples from 3 normal dogs. B) serum sample from 1 normal dog. C) Serum samples from 7 dogs with skin diseases. a,b) Columns with different superscripts differ (P<0.05). F, Female; M, Male; FT₄, Serum free thyroxine concentrations; TSH, Serum thyroid stimulating hormone concentrations; TgAA, thyroglobulin autoantibody; NT, Not tested; Cast, Castrated male; Ovx, Ovariohysterectomized female.
lot patterns were disappeared after pretreatment with sufficient amount of intact cTg. Mild proteolytic treatment of native cTg was performed according to the method of Saboori et al. [29]. It has been reported that reduction and alkylation of Tg destroyed its ability to bind to autoantibodies from human patients with thyroid diseases [21]. Saboori et al. [29] concluded that mild proteolytic treatment of native Tg released conformational peptides that could be used for identifying antibody-binding regions, and the post-translational modifications that may affect antigenicity would also be retained. Therefore, these results indicate that TgAA recognizes conformational epitopes on the Tg molecule and the epitopes recognized by TgAA depend upon individual dogs with hypothyroidism. Sera from normal dogs as well as dogs with hypothyroidism reacted to several tryptic peptides with molecular weight ranging from 15 to 20 kDa and 40 kDa. However, it is unknown whether these peptides could be associated with pathogenicity. TgAA must recognize antigens associated with proteolytic processing of Tg in the thyroid gland.

The prognostic significance of the presence of autoantibodies to thyroglobulin has been unclear. Evidence for progressive lymphocytic thyroiditis in dogs includes 2 of 11 Cocker Spaniels at 18 months [15] and 2 of 10 Borzois [7] followed for 2 years, which progressed to abnormal function tests or clinical signs of hypothyroidism, respectively. In the recently completed 12-month prospective study, 4% of 171 TgAA positive dogs developed clear evidence for classic functional hypothyroidism and 13% had laboratory evidence to suggest progression to subclinical hypothyroidism [13]. These findings suggest that the annual rate of progression of the disease is low and in individuals. It has been shown that the presence of thyroid pathogenic changes in dogs does not necessarily mean the presence of thyroid functional abnormalities [20, 23]. Thus, without routine biopsies, progression levels of the disease can only be
inferred from results of thyroid antibody studies. The present methods for detection of TgAA are susceptible to interference by the potential for false-positive results. In human, a 7.2% yearly incidence of clinical hypothyroidism in thyroid antibody positive patients and 26% in patients with initially raised TSH concentrations has been reported [11]. It seems that the epitopic recognition by TgAA in human patients with thyroid autoimmune disease differs from that in healthy people with thyroid antibodies, and this difference has been proposed to be useful to predict the onset of thyroid disease in humans [5, 28]. Many investigators have been using intact cTg as antigen for the measurement of thyroglobulin autoantibodies, so that some of the positive dogs must have shown false-positive [9, 16, 17, 31]. Our findings show the possibility of the specific methods for detection of pathogenic TgAA without false-positive results. It is needed to determine the dominant epitopes of TgAA in the sera from dogs with hypothyroidism, and to develop the assay into predictive diagnosis of hypothyroidism by using the epitopic information.

Two of the dogs with clinical symptoms of hypothyroidism had low TgAA titers in the present study. Complete disorder of thyroid functions attributable to the long-lasting hypothyroidism may lead to a decline in antigenic substances resulting in reduced TgAA production. In a recent study, canine TgAA titers were found to decline during L-thyroxine treatment [8]. Alternatively as proposed in a study with humans receiving L-thyroxine substitution therapy, there could be a reduction of antigenic substances through a decreased stimulation of thyroid tissue by circulating TSH [27].

This must be the first study to determine the recognition pattern of circulating TgAA in hypothyroid dogs. The findings that different recognition patterns of cTg reacted to antisera from hypothyroid dogs might indicate serological markers of the disease, if their pathogenic epitopes can be
defined. There is a possibility that B cells of individual dogs with hypothyroidism recognize different epitopes of cTg. The Graves’ disease Tg and Tg from human patients with thyroid carcinoma have a structure differing somewhat from Tg of normal individuals [18]. It is hypothesized that dogs lineage predisposing to hypothyroidism has an abnormal structure of Tg. These Tgs may be processed differently by proteases in the thyroid glands, and therefore antigen-presenting cells present different epitopes to T cells. Further investigation of the amino acid sequence of cTg may lead to a better understanding of the characterization of these epitopes. These epitopes must be compared between dog lineages predisposing to hypothyroidism.

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REFERENCES


