Development, characterization and clinical validation of new sensitive immunofluorometric assay for the measurement of serum thyroglobulin

Desenvolvimento, caracterização e validação clínica de um novo ensaio sensível para a dosagem da tiroglobulina sérica

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ABSTRACT

Objective: In the last decade, data published stressed the role of highly-sensitive thyroglobulin (Tg) assays in the follow-up of differentiated thyroid carcinoma (DTC) patients. The present study describes a new, highly-sensitive Tg assay, compares it with an available commercial assay, and validates it in the follow-up of DTC patients. Subjects and methods: The immunofluorometric high-sensitivity Tg assay is based on monoclonal and polyclonal antibodies produced at our laboratories. It was validated in 100 samples of 87 patients with DTC submitted to total thyroidectomy, 87% of whom also received radioiodine. For correlation, all samples were also tested using a commercial Tg assay (Beckman Access) with functional sensitivity (FS) of 0.1 ng/mL. Results: The new method showed FS of 0.3 ng/mL. The correlation between the two methods was good (r = 0.74; p < 0.0001). The diagnostic sensitivity was 88.9%, and it was increased to 100% when combined with neck US. Conclusion: This new, high-sensitivity Tg assay presented a good correlation with Beckman Access assay and with the clinical outcome of the patients. The continuous availability of a validated assay is an additional advantage for long term follow-up of DTC patients.

Keywords
Differentiated thyroid cancer; thyroglobulin; highly-sensitive assay

RESUMO

Objetivo: Na última década, estudos mostraram a importância dos ensaios de tiroglobulina (Tg) com melhor sensibilidade funcional no seguimento dos pacientes com carcinoma diferenciado de tiroide (CDT). Neste estudo, descrevemos o desenvolvimento de um novo ensaio de Tg de alta sensibilidade, que foi validado no seguimento de pacientes com CDT e correlacionado com um ensaio comercialmente disponível. Sujeitos e métodos: O ensaio imunofluorométrico de Tg baseia-se em anticorpos, um monoclonal e um policlonal desenvolvidos em nosso laboratório. Avaliamos 100 amostras de soro de 87 pacientes com CDT submetidos à tiroidectomia total, sendo que 87% deles também receberam 131I. ATg foi dosada também em ensaio comercial (Beckman Access). Resultados: A correlação entre os dois métodos foi de 0,74 (p < 0,0001). O novo ensaio mostrou uma sensibilidade funcional de 0,3 ng/mL. A sensibilidade diagnóstica foi de 88,9%, que aumentou para 100% quando associada ao ultrassom cervical (US). Conclusão: O novo método de dosagem de Tg mostra boa correlação com o ensaio comercial Beckman Access e com a evolução clínica dos pacientes. O novo ensaio será fundamental no seguimento dos nossos pacientes com CDT.

Descritores
Carcinoma diferenciado de tiroide; tiroglobulina; ensaio de alta sensibilidade
INTRODUCTION

Thyroglobulin (Tg) is a glycoprotein secreted exclusively by thyrocytes into the lumen of thyroid follicles. It is the precursor of the thyroid hormones and the major component of the thyroid colloid. The main form of Tg is a 660-kDa dimer composed of two identical 330-kDa monomers. Human Tg gene is located on the long arm of chromosome 8, encoding 2,767 amino acid residues that, when transcribed, originate the monomer (1). Tg goes to extensive post-translational modifications, including glycosylation, iodination and polymerization, all contributing to marked heterogeneity within the colloid. Reduction and degradation also lead to the formation of smaller polypeptides (2). Formation of thyroid hormones occurs within the Tg molecule, and they are secreted in the circulation after Tg contained in the colloid undergoes enzymatic degradation, following a micropinocytosis process by the thyrocytes. This process is under direct TSH control, but degradation is not the only fate of Tg. It can also be recycled back into the colloid, or secreted via transcytosis. This late process is mediated by binding to megalin, a member of the LDL receptor family, and it is one of the mechanisms responsible for the presence of significant levels of circulating Tg (2). This pathway is now recognized as a control mechanism of thyroid hormone release (3). Binding of Tg to megalin depends on conformation, and the degree of iodination, which has conformational consequences, is one of the mechanisms that alter serum Tg levels independently of TSH stimulation (4).

The first practical and sensitive method for serum Tg measurement was described in 1973 by Van Herle and cols. (5). It was a radioimmunoassay that used radioactive iodine-labeled human Tg and an antibody generated in rabbits against the same Tg preparation used for labeling. Since then, Tg measurement has been used for the evaluation of the presence of thyroid tissue, mainly in the follow-up of patients diagnosed with differentiated thyroid cancer (DTC) (6,7). Evolution of the methodology was constant in these almost four decades, and now we have access to assays that are more sensitive, fast, automated, precise and commercially available. Nevertheless some chronic problems still plague Tg measurement and can be divided in two groups: one derived from the heterogeneity of circulating Tg, and the other derived from the possible presence of endogenous anti-mouse IgG (HAMA) and anti-Tg antibodies (anti-Tg). In the first case, the problem is related to the complex metabolic pathway of Tg, as described above. Polyclonal and monoclonal antibodies used in immunassays recognize Tg epitopes present in the immunization preparation used to generate them, and these forms may not be predominant in a serum sample. Recent studies have shown that available assays may differ significantly in the levels of Tg detected (8,9), and the search for the ideal assay is still a challenge (10).

The presence of HAMA and/or anti-Tg antibodies is the other conundrum of Tg assays. HAMA is a potential problem for all immunometric assays, since most of them are based on monoclonal antibodies produced in mice. In the case of Tg assays, as in most other immunometric assays, the presence of HAMA can lead to false-positive (most of the time), or false-negative results (11,12). The potential presence of HAMA is still a challenge, but accumulated knowledge has paved the way to deal adequately with the phenomenon most of the time (13). The presence of endogenous anti-Tg antibodies is, in Tg assays, an even more complex problem, generating false-negative results. The high frequency of anti-Tg antibodies in the general population, particularly in patients with DTC, and the difficulty of standardizing assays to detect anti-Tg antibodies, makes the problem even more complex (14-17). Competitive assays for Tg do not seem to be too sensitive to this problem, but they are no longer used in clinical laboratories, being available only in few research laboratories (15).

In this paper, we describe the development of a sensitive immunofluorometric Tg assay based on a rabbit polyclonal antibody and a mouse monoclonal antibody anti-Tg. We describe its characterization and clinical validation in serum samples of patients with DCT with negative and positive anti-Tg antibodies. Correlation with a commercial automated immunometric assay is also provided.

PATIENTS

We evaluated this new assay in 87 DTC patients who were followed up by a single team of physicians at the associated Thyroid Disease Centers at the Division of Endocrinology, Department of Medicine, Escola Paulista de Medicina, Universidade Federal de São Paulo and at the Instituto Israelita de Ensino e Pesquisa Albert Einstein, both in Sao Paulo, Brazil. All the diag-
nostic procedures were performed in accordance with
the guidelines determined by the local Ethics Com-
mittee. Written informed consent was obtained from all
the patients.

All patients had been treated with total or near-
total thyroidectomy and 76/87 had also received 131I
ablation or treatment (30 to 750 mCi) for the thyroid
remnant. Patients were 78 women and 9 men, with
mean age at diagnosis of 40 years old (19 to 79 years
old), 79 with papillary thyroid carcinoma (PTC), and 8
with follicular thyroid carcinoma (FTC). Mean time of
follow-up was 52 months (21 to 80 months) after Tg
measurements. Patients were classified, for better data
analysis, as follows:
1) Group 1 (n = 42): 42 patients ‘without evidence of
disease or thyroid tissue remnant’, when there was
no evidence of disease or thyroid tissue remnant,
either clinically or in laboratorial and imaging tests
(stimulated Tg levels < 1.0 ng/mL, negative whole
body scan - WBS -, and neck US negative for suspi-
cious images).
2) Group 2 (n = 17): 17 patients ‘without evidence of
disease and with thyroid tissue remnant’, when they
had positive thyroid bed uptake on diagnostic or
post-therapeutic WBS with negative US, and stimu-
lated serum Tg < 10 ng/mL.
3) Group 3 (n = 15): 15 patients ‘with metastasis’,
when there was evidence of disease in cytological or
histological analysis, or thyroid bed uptake in WBS.
4) Group 4 (n = 7): 7 patients ‘with metastasis and pos-
itive anti-Tg’, when there was evidence of disease in
cytological or histological analysis, or thyroid bed
uptake in WBS with positive serum test for anti-Tg.

A total of 100 measurements were analyzed, as
12/87 patients had more than one Tg measurement in
different periods of follow-up.

MATERIALS AND METHODS

“In house” Tg assay

Polyclonal antibodies anti-human thyroglobulin were
produced at our laboratory in rabbits immunized
with thyroglobulin by the multiple injection protocol
(18,19). The thyroglobulin preparation used for immu-
unization was obtained from human thyroid gland
collected at surgery from patients submitted to sub-
total thyroidectomy for treatment of Graves’ disease
(19,20). Purification was based on ammonium sulfu-
te precipitation (19,20), and the antibody (rabbit A2)
was used in a final dilution of 1/15,000 in Tris-HCL
50 mM, pH 7.5, with 0.5% BSA and 0.05% of bovine
gammaglobulin.

Monoclonal antibodies (mAb) anti-human thyro-
globulin were obtained at our laboratory from hybrid-
ons produced from spleen cells collected from mice
immunized with the same thyroglobulin preparation
used for the polyclonal antibody production. Stan-
dard immunization and fusion protocols (21) were
used. Screening (using human thyroglobulin > 97%
pure purchased from Scripps Laboratories, San Diego,
CA, USA), selection, ascites production, purification,
and IgG subtyping followed the same methodology
described previously (22). Titer and specificity studies
were conducted, and the cell line D6P2, an IgG1 kapp,
a was selected.

Monoclonal antibodies anti-rabbit IgG (D4P4,
IgG1 kappa) were produced at our laboratory by the
same protocol described for the thyroglobulin mAb
production. Mice were immunized with rabbit serum
IgG, > 95% pure, purchased from Sigma-Aldrich, St.
Louis, MO, USA. This mAb was biotinylated with N-
hydroxysuccinimide-biotin (NHS-biotin, Pierce, Rock-
ford, IL, USA) (23). After dialysis purification, the bio-
tinylated mAb was used at a final dilution of 1/2,000
in the same buffer used for the rabbit anti-Tg mAb with
the addition of 1% normal mouse serum.

Europium-labeled streptavidin (Perkin Elmer, Turku,
Finland) was used at a 1/2,000 dilution and fluorometry
measured using a time-resolved fluorometer (Perkin
Elmer, Turku, Finland). Standard curve was produced
by serial dilution of human thyroglobulin reference
standard CRM 457 (Institute for Reference Materials
and Measurements, European Comission, Geel, Bel-
gium) in a pool of sera from patients with previous un-
detectable levels of thyroglobulin and anti-thyroglobu-
lin antibodies. This serum pool was previously treated
with charcoal, centrifuged, and filtered through a 0.45-
µm filter (Millipore, USA). The standard curve ranged
from 400 to 0.78 ng/mL.

Serum samples from patients from our Thyroid Dis-
eases Centers were obtained by venipuncture and after
serum separation, stored at -20°C until analysis.

“In house” Assay Protocol

The wells of microtiter plates (96-well FluoroNunc,
Nunc, Roskilde, Denmark) were coated with 200 µL of
a solution of the anti-Tg mAb (D6P2), 10 µg/mL in
20 mM PBS, pH 7.4. After an overnight incubation at
4°C, wells were washed three times with washing buffer (PBS, pH 7.4 with 0.05% Tween 20) and treated with 200 µL of Tris-HCl 50 mM, pH 7.75, containing 0.5% BSA and 0.05% bovine gammaglobulin, for 1 hour at 37°C. Plates were emptied by inversion and after a new washing procedure, 100 µL of sample or standard curve were pipetted in duplicate, followed by 100 µL of the polyclonal rabbit anti-Tg antibody at a final dilution of 1/5,000 in Tris-HCl 50 mM pH 7.75 with 0.5% BSA and 0.05% bovine gammaglobulin. Plates were shaken in a horizontal shaker for 1h at room temperature, sealed, and incubated for 48h at 4°C.

After the incubation and new washing procedure, 200 µL of a 1/2,000 dilution of the biotinylated anti-rabbit IgG mAb was added, plates agitated for 2h at room temperature, washed, and 200 µL of a 1/2,000 dilution of europium-labeled streptavidin (PerkinElmer, Turku, Finland) was added. After 30 minutes of incubation under agitation, plates were washed and 200 µL of fluorescent solution (PerkinElmer) were added, with time-resolved fluorescence read after 5 minutes of agitation.

**Tg Beckman Access immunoassay**

The Tg Access assay (Beckman Coulter, Fullerton, CA, USA) is a one-step “sandwich” assay. A sample is added to a reaction vessel along with a mixture of four biotinylated monoclonal anti-Tg antibodies, streptavidin-coated paramagnetic particles, and a monoclonal anti-Tg antibody alkaline phosphatase conjugate. The biotinylated antibodies bind to the solid phase and serum thyroglobulin binds to these antibodies, while the conjugate antibody reacts with a different antigenic site on the thyroglobulin molecules. After incubation in a reaction vessel for 42 minutes, materials bound to the solid phase are held in a magnetic field while unbound materials are washed away. A chemiluminescent substrate, Lumi-Phos** 530 is added to the reaction vessel and light generated by the reaction is measured with a luminometer. Light production is directly proportional to the concentration of thyroglobulin in the sample. The amount of Tg in the sample is determined by comparison with a stored, multipoint calibration curve.

**RESULTS**

**Characteristics of the assays**

“In house” assay: analytical sensitivity was 0.07 ng/mL; intra-assay CV was 7.2% for a pool with a mean value of 0.67 ng/mL, 3.7% for a pool with a mean value of 2.9 ng/mL, and 4.9% for a pool with a mean value of 158.0 ng/mL. Inter-assay CV was 15.8%, 12.5%, and 10.8% for the same pools. Functional sensitivity (FS) of the assay was 0.3 ng/mL.

Beckman Access assay: analytical sensitivity was 0.01 ng/mL; intra-assay CV was 1.4% for a pool with a mean value of 4.2 ng/mL, 1.4% for a pool with a mean value of 21.6 ng/mL, 4.4% for a pool with a mean value of 130.4 ng/mL, and 2.0% for a pool with a mean value of 344.7 ng/mL. Inter-assay CV was 1.7%, 1.8%, 4.9% and 4.0% for the same pools. Total CV was 4.9% for a pool with a mean value of 4.2 ng/mL, 5.2% for a pool with a mean value of 5.3 ng/mL, 6.4% for a pool with a mean value of 34.0 ng/mL, 3.8% for a control with a mean value of 42.5 ng/mL, and 4.6% for a control with a mean value of 213.0 ng/mL. FS of the assay was 0.1 ng/mL.

**Clinical data**

Correlation between both methods in all 100 measurements (87 patients) was considered good with $r = 0.74$ ($p < 0.0001$) and is shown in figure 1.

The analysis of the 42 patients ‘without evidence of disease or thyroid tissue remnant’ (Group 1), has shown that Tg was undetectable in 40/42 patients using the Beckman Access assay, and in 36/42 with our “in house” method. Therefore, a total of 7 of these 42 patients had detectable levels of Tg in one or both assays, but they were in low levels: maximum Tg values were 0.9 ng/mL with Beckman Access, and 0.8 ng/mL.
mL with our assay (Figure 2). During the follow-up of these 7 patients, Tg became undetectable in 5 of them.

The analysis of the 17 patients with evidence of ‘thyroid tissue remnant’ (Group 2) showed that 16/17 had detectable Tg with the “in house” methodology, and 11/17 with the Beckman Access assay, showing that these assays are very sensitive for the presence of thyroid tissue. From those 16 patients with detectable Tg in our method, 7 had received 30 mCi of radioiodine for thyroid ablation, and 7 had not received radioiodine. All 17 patients were considered without evidence of disease during the period of follow-up of at least 40 months (Table 1).

The evaluation of 15 patients ‘with metastasis and negative TgAb antibodies’ (Group 3) showed that Tg was detectable in 13/15 patients with both methods, a diagnostic sensitivity of 88.9% with both methods, which increased to 100% when combined with neck US. The correlation between methods in this group of patients was also good ($r = 0.77$ and $p < 0.0001$) (Figure 3).

On the other hand, Tg was undetectable with both methods in all 7 patients with ‘metastasis and positive anti-Tg’ (Group 4), showing, as expected, antibody interference.

Our method showed a 42.1% positive predictive value (PPV), 94.7% negative predictive value (NPV) and 68.4% accuracy when all measurements were evaluated (Table 2). When patients with evidence of thyroid tissue remnant were excluded, a 68.4% positive predictive value (PPV), a 94.7% negative predictive value (NPV), and 86% accuracy were observed (Table 3). These data show that our method also detects the presence of Tg from minimal thyroid remnants.

### Table 1. Clinical and laboratory data of 17 patients with evidence of ‘thyroid tissue remnant’

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender</th>
<th>Age at diagnosis (years)</th>
<th>TNM</th>
<th>$^{131}$I activity before measurement of Tg (mCi)</th>
<th>Tg access</th>
<th>Tg in house</th>
<th>US</th>
<th>WBS</th>
<th>Follow-up (months)</th>
<th>Actual status</th>
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<td>1</td>
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<td>26</td>
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<td>TB</td>
<td>79</td>
<td>NED</td>
</tr>
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<tr>
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<td>$&lt;0.3$</td>
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<td>TB</td>
<td>40</td>
<td>NED</td>
</tr>
<tr>
<td>4</td>
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<td>24</td>
<td>T3N0M0</td>
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<td>0.6</td>
<td>0.6</td>
<td>neg</td>
<td>TB</td>
<td>72</td>
<td>NED</td>
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<tr>
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<td>0</td>
<td>0.6</td>
<td>1.0</td>
<td>neg</td>
<td>TB</td>
<td>58</td>
<td>NED</td>
</tr>
<tr>
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<td>T1N0M0</td>
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<td>TB</td>
<td>49</td>
<td>NED</td>
</tr>
<tr>
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<td>1.5</td>
<td>3.4</td>
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<td>TB</td>
<td>58</td>
<td>NED</td>
</tr>
<tr>
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<tr>
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<td>350</td>
<td>$&lt;0.1$</td>
<td>0.4</td>
<td>neg</td>
<td>TB</td>
<td>42</td>
<td>NED</td>
</tr>
</tbody>
</table>

F: female; M: male; neg: negative; WBS: whole body scan; TB: thyroid bed; NED: none evidence of disease.
DISCUSSION

First, why develop a new Tg assay? Besides having the know-how for this development, we were mostly driven by the possibility of having access to the same assay for a long period of time. As seen, commercial assays are constantly modified in order to improve performance, which is a good tendency, but differences in the recognition of Tg molecules can vary along the way. Besides, development is costly and it is cheaper to maintain an “in house” diagnostic routine test. Also, the processes of Tg formation within follicular cells can be modified in thyroid neoplasms, resulting in conformational changes in the secreted Tg molecules (25). Considering the conformational nature of Tg epitopes (26), probably IMA methods detect different and more restricted Tg isoforms, resulting in wider between-method bias when measuring tumor-derived Tg as compared with non-tumor Tg (8).

The American, European and Brazilian Thyroid Association guidelines suggest that a patient with DTC can be considered free of disease when there is no clinical and no imaging evidence of tumor, and Tg is undetectable during TSH suppression and following TSH stimulation (27-29). However, some authors have shown that TSH stimulation is not necessary when using second generation Tg assays with functional sensitivity of 0.1 to 0.3 ng/mL (30-32).

Our method has functional sensitivity (FS) of 0.3 ng/mL and has shown good sensitivity and high NPV. The 42.1% of PPV was probably caused by patients with evidence of thyroid tissue remnant, and because we arbitrarily defined the Beckman Access method as the ‘gold standard’. Our performance would be much better if it was the other way around: with our method as the ‘gold standard’, because 16/17 patients with evidence of thyroid tissue remnant had detectable Tg with our methodology, and 12/17 with the Beckman Access Assay. In this group of patients with evidence of thyroid tissue remnant, 7/17 have not received radioiodine for thyroid ablation and 7/17 have received 30 mCi radioiodine for thyroid ablation, showing that our assay is very sensitive for the presence of thyroid tissue, probably due to the use of polyclonal antibodies. All 17 patients had negative US and at least 40 months of follow-up without evidence of disease. Most importantly, Tg values decreased in most patients or remained at the same level in others. Therefore, clinicians should be aware of this feature,

| Table 2. Sensitivity, specificity, VPP, VPN and accuracy of both methods in all measurements |
|----------------------------------------|-----------------|----------------|-----------------|-----------------|-----------------|
| Method                  | Sensitivity     | Specificity  | PPV           | VPN           | Accuracy        |
| Beckman                 | 88.9%           | 77.6%        | 55.2%         | 95.7%         | 80.3%           |
| In house                | 88.9%           | 62.1%        | 42.1%         | 94.7%         | 68.4%           |

| Table 3. Sensitivity, specificity, VPP, VPN and accuracy of both methods excluding patients with evidence of thyroid remnants |
|----------------------------------------|-----------------|----------------|-----------------|-----------------|-----------------|
| Method                  | Sensitivity     | Specificity  | PPV           | VPN           | Accuracy        |
| Beckman                 | 86.7%           | 95.2%        | 86.6%         | 95.2%         | 93%            |
| In house                | 86.7%           | 85.7%        | 68.4%         | 94.7%         | 86%            |
because many patients with thyroid tissue remnant will have Tg measured with assays with better functional sensitivity. A Tg ‘trend’ may be more important than a single measurement, because rising Tg values over time raise the suspicion of growing thyroid tissue or recurrence of disease (27).

The new method detected almost all patients with metastasis in patients with negative anti-Tg and suppressed TSH, and reached 100% sensitivity when combined with neck US and cytology.

Interference caused by heterophilic antibodies (HAMA) is a potential problem for all assays employing IMA methodology and mouse monoclonal antibody reagents (11). HAMA interference arises when antibodies that recognize murine proteins are present in the patient serum and interact with the capture and/or labeled monoclonal antibody reagents. These reactions simulate the presence of thyroglobulin in the specimen, and can create a falsely elevated result and, rarely, a false-negative result (12,33). Our assay uses antibodies derived from rabbit (polyclonal) and mouse (monoclonal), which potentially decrease the interference by heterophilic antibodies, because it is more unusual that one patient has antibodies reacting against immunoglobulins from two different species.

As expected, patients with positive anti-Tg antibodies are still a challenge for immunometric Tg assays. The interference caused by anti-Tg antibodies remains the most serious problem limiting the clinical utility of Tg testing, and we did not overcome that for now. In our study, 7 patients with metastasis and positive anti-Tg had undetectable Tg with both assays. Having continuous access to the same methodology, we will be able to study alternatives in order to deal with the interference of these antibodies in our assay. Until now, to prevent interference by TgAb, we and others have developed qualitative RT-PCR assay to amplify mRNA Tg present in circulating thyroid cells from patients with DT, allowing differentiation between patients “free of disease” and those with metastases (34,35). Therefore, mRNA Tg could be an appropriate molecular marker for the follow-up of patients with thyroid carcinoma, especially in patients with positive TgAb.

In conclusion, this new method of Tg measurement could represent an improvement in terms of sensitivity when compared with the most widely employed assays in our country. Moreover, the use of antibodies from two different species in this new assay possibly decreases the potential interference of heterophilic antibodies.

Further, we will be able to rely on the same method in our clinical setting for long term follow-up of DTC patients and study alternatives to deal with the interference of anti-Tg antibodies.

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REFERENCES
8. Spencer CA, Bergoglio LM, Kazarosyan M, Fatemi S, LoPresti JS. Clinical impact of thyroglobulin (Tg) and Tg autoantibody method differences on the management of patients with differentiated thyroid carcinomas. J Clin Endocrinol Metab. 2003;90:5998-75.

15. Spencer C, Petrovic I, Fatemi S. Current thyroglobulin autoantibody (TgAb) assays fail to detect interfering TgAb that can result in the reporting of falsely low/undetectable serum TgIMA values for patients with differentiated thyroid cancer. J Clin Endocrinol Metab. 2011;96:1283-9.


