Performance and Specificity of 6 Immunoassays for TSH Receptor Antibodies: A Multicenter Study

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Keywords

Thyroid-binding inhibitory immunoglobulins · TSH receptor stimulating antibodies · TSH receptor blocking antibodies · Bioassays · Binding assays · Specificity

Abstract

Background: The measurement of TSH receptor (TSHR) antibodies is warranted for diagnosis of Graves' disease (GD). **Objective:** The performance, detection sensitivity, and specificity of 6 TSHR immunoassays were compared. **Methods:** Two bioassays and 4 binding assays (Kronus, Immulite, Kryptor, Dynex) were compared in a dilution study performed in patients with autoimmune thyroid disease. Both bioassays were compared to 2 binding assays using stimulatory (M22) and blocking (K1–70) monoclonal antibody (MAb) mixtures. **Results:** Thirty samples from stimulatory (TSAb)-positive/blocking (TBAb)-negative patients with GD were diluted serially and measured in all assays. Samples were positive until dilution 1:2,187 in the TSAb bioassay, 1:81 in the

Immulite ($p < 0.002$ vs. bioassay) and Kronus ELISA ($p = 0.039$) assays, and 1:27 in the Kryptor and Dynex ELISA ($p < 0.001$ vs. bioassay). Ten samples from TBAb-positive/TSAb-negative patients with GD or Hashimoto's thyroiditis were positive in all binding assays. None of the binding assays differentiated between TSAb and TBAb. Mixtures of 100% K1–70 (200 ng/mL), 80% K1–70 + 20% M22, 60% K1–70 + 40% M22, 40% K1–70 + 60% M22, 20% K1–70 + 80% M22, and 100% M22 (20 ng/mL) tested positive in both Immulite (26.4, 20.2, 15.2, 10.5, 6.3, 2.00 IU/L) and Kronus assays (27.1, 23.3, 19.3, 12.0, 5.7, 2.2 IU/L). These MAb mixtures were tested in the TBAb bioassay and showed 82, 61, 24 (negative), –26 (negative), –77 (negative), and –95% (negative) inhibition, respectively. **Conclusions:** The sample dilution study showed higher detection sensitivity for the TSAb bioassay, and the antibody mixture study demonstrated exclusive specificity of the bioassays over all automated and ELISA binding assays.

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Introduction

Antibodies (Ab) to the thyroid-stimulating hormone receptor (TSHR) may mimic [1–3] or block [4] the action of TSH or be functionally neutral [5]. TSHR stimulating Ab (TSAb) are responsible for many of the clinical manifestations of Graves' disease (GD) and are specific biomarkers [6–10] of this autoimmune thyroid disease (AITD). On the other hand, TSHR blocking Ab (TBAb) can also be found in AITD patients and may contribute to the hypothyroidism or atypical Hashimoto's thyroiditis (HT). Ever since the different functional activities of TSHR Ab have been recognized it has been suspected that certain patients might contain both TSAb and TBAb, and that this might explain the spectrum of clinical presentations. Indeed, recent evidence has proven that a patient can have both TSAb and TBAb by isolating monoclonal antibodies (MAb) with stimulatory and blocking activity from the lymphocytes of the same patient [11]. The measurement of TSAb and TBAb has potential clinical implications in the differential diagnosis of AITD, in predicting the outcome of GD after antithyroid drug treatment, in evaluating the risk of extrathyroidal manifestations of GD during pregnancy, and in predicting the likelihood of fetal/neonatal hyper- or hypothyroidism [10, 12]. TSHR Ab can be measured with either a bioassay or a binding assay. However, bioassays have the advantage of indicating not only the presence of Ab but also their functional activity and potency.

Since no studies that compare the performance of TSHR Ab immunoassays are available, we aimed to measure the lowest possible Ab concentration in several immunoassays and to compare the performance of ELISA and automated assays. FDA-cleared and/or validated functional cell-based bioassays for the measurement of TSHR Ab, whose analytical performance has been published [13–15], were used for assay comparison. In the second part of this work, 2 human TSHR MAb were tested on an automated assay and an ELISA assay with regard to specific TSHR Ab functionality. Thus, in this study both patient sera containing polyclonal Ab and human MAb were applied.

Material and Methods

A total of 40 well-characterized patients with AITD were enrolled in this study. Samples were collected from 3 institutions (Johannes Gutenberg University [JGU] Medical Center, Mainz, Germany; Medical University of Białystok, Poland; and UZ Leuven, Belgium) while measurements were performed in 2 laboratories

(JGU and Prof. Wüster, Mainz). HT was defined as a serum level of anti-thyroid peroxidase Ab above the reference range with or without increased serum concentration of anti-thyroglobulin Ab, a hypoechoic appearance at thyroid ultrasound, and euthyroidism or hypothyroidism. GD was defined as positive TSHR Ab, suppressed baseline TSH, elevated free thyroid hormones (free triiodothyronine, fT3 and/or free thyroxine, fT4), and enhanced vascularization at thyroid ultrasound ("thyroid inferno"), with or without clinically manifestations of orbital disease. The study protocol was approved by the JGU Ethics Committee and was carried out in accordance with the ethical guidelines of the Helsinki Declaration. Informed consent was obtained from all participants investigated in the study.

Thyroid-Related Hormones and Antibodies

Serum concentrations of TSH, fT3, fT4, anti-thyroglobulin, and anti-thyroid peroxidase Ab were evaluated using electrochemiluminescence immunoassays (CLIA; Abbott, Wiesbaden, Germany) according to the manufacturer's instructions. For the diagnosis of GD, serum samples were measured for thyroid-binding inhibitory immunoglobulins (TBII) with the Cobas e411 analyzer, Elecsys (Roche Diagnostics, Mannheim, Germany). For the dilution analysis, TBII concentrations were measured with 4 different binding assays: Kronus (Star, ID, USA), Immulite 2000 XPi (Siemens, Erlangen, Germany), Kryptor (ThermoFisher, Hennigsdorf, Germany), and DS2 (Dynex, TECOMedical, Bünde, Germany). All binding assays were performed according to the manufacturer's instructions.

TSHR Stimulating and Blocking Antibody Bioassays

Serum TSAb activity was measured with a chimeric TSHR bioassay (Quidel Corp., San Diego, CA, USA) according to manufacturer's instructions [13, 14, 16]. TSAb activity was reported as percentage of specimen-to-reference ratio (SRR%) (cutoff SRR% <140). Serum TBAb activity was measured as described previously [15, 17]. Blocking activity was defined as percent inhibition of luciferase expression relative to induction with bovine TSH alone (cutoff <40% inhibition). All bioassay measurements were done in duplicate. Both bioassays have been validated [13–15], and the standardization of the TSAb bioassay was previously reported [16].

Serial Dilution Analysis

The TSAb bioassay was compared with the TBII assays using 30 serum samples from 30 untreated patients with GD. Serial 1:3 dilutions were performed on each serum sample (200 µL) in duplicate into human TSAb-negative control serum (400 µL collected from a euthyroid, healthy subject devoid of thyroid, autoimmune, and oncological disease) up to a final dilution of 1:81. For samples still positive at 1:81, the dilution was extended to a final dilution of 1:243, 1:729 or 1:2,187.

Detection of Various Human TSHR MAb Mixtures with Stimulating (M22) and Blocking MAb (K1–70) Activities

MAb mixtures were diluted in control serum (collected from a healthy subject devoid of thyroid and autoimmune disease) with 20 ng/mL of TSAb M22 (Kronus) and 200 ng/mL of TBAb K1–70 (Kronus) at different ratios. Mixtures of 100% K1–70 (200 ng/mL), 80% K1–70 (160 ng/mL) + 20% M22 (4 ng/mL), 60% K1–70 (120 ng/mL) + 40% M22 (8 ng/mL), 40% K1–70 (80 ng/mL) + 60% M22

(12 ng/mL), 20% K1-70 (40 ng/mL) + 80% M22 (16 ng/mL), and 100% M22 (20 ng/mL) were prepared and measured in the TBAb bioassay and Immulite and Kronus assays following the manufacturers' instructions.

Statistical Analysis

Data of all figures are presented as mean $\pm$ SEM. Data of thyroid-related hormones and Ab are shown as median and 25th and 75th percentiles. All experimental data were analyzed with GraphPad Prism Software, Inc. (version 5.04), San Diego, CA, USA. Positivity rates for each dilution were compared between TSAb and the different binding assays (TBII) by the McNemar test. Correlations between the TSAb bioassay and the 4 binding assays were assessed with the Spearman correlation coefficient and the corresponding test for zero correlation. All p values were 2-sided and considered significant when $p \leq 0.05$.

Results

The demographic and serological data of all 40 investigated patients with AITD are summarized in Table 1. Thirty well-characterized, untreated, hyperthyroid TSAb-positive/TBAb-negative patients with GD were included as well as 10 euthyroid (on levothyroxine) TBAb-positive/TSAb-negative patients with either GD or HT.

Assay Performance

The number of positive samples in all 30 hyperthyroid patients with GD, undiluted and at each dilution step (1:3 to 1:2,187), measured in the TSAb bioassay and the Immulite, Kryptor, and 2 Kronus and Dynex ELISA assays are shown in Figure 1. The 30 undiluted serum samples from patients with GD were all positive in the TSAb bioassay, Kronus ELISA, and Immulite assay. However, only 25/30 (83.3%) and 24/30 (80%) were positive with the Kryptor and Dynex ELISA, respectively ($p = 0.063$ and $p = 0.031$ vs. bioassay). The results of the 30 undiluted GD samples measured by the TSAb bioassay positively correlated with those measured by the Kronus ELISA ($r = 0.538$, $p = 0.002$), Immulite ($r = 0.779$, $p < 0.001$), Kryptor ($r = 0.510$, $p = 0.004$), and Dynex ($r = 0.613$, $p < 0.001$).

In the dilution analysis, samples were positive until dilution 1:2,187 in the TSAb bioassay, 1:81 in the Immulite ($p < 0.002$ vs. bioassay) and Kronus ELISA ($p = 0.039$) assays, and 1:27 in the Kryptor and Dynex ELISA ($p < 0.001$ vs. bioassay). The TSAb bioassay and TBII binding assay median and interquartile (25th and 75th percentile) values for 40 undiluted serum samples from patients with either GD or HT are shown in Table 2. Overall, the sample dilution study showed higher detection sensitivity for the TSAb bioassay.

Table 1. Demographic and serological data of the 40 investigated patients with autoimmune thyroid disease and positive thyroid-binding inhibitory immunoglobulins (TBII)

	Untreated hyperthyroid patients with Graves' disease	Patients with either Graves' disease or Hashimoto's thyroiditis on levothyroxine
Patients, N	30	10
Gender (F/M), n	23/7	10/0
Age, years	41.4 (30.6–53.1)	37 (34.3–54.3)
TBII Cobas (<1.75 IU/L)	6.4 (3.6–14.8)	34.3 (10.8–40)
TSAb	positive	negative
TBAb	negative	positive
TSH (0.4–4.9 mU/L)	0.01 (0.01–0.01)	0.09 (0.01–2.2)
fT3 (2–4.4 pmol/L)	6.5 (4.7–8.2)	3.9 (3.2–5.7)
fT4 (0.9–1.7 ng/dL)	1.9 (1.6–2.4)	1.2 (1–2)
Tg Ab (<4.1 IU/mL)	5.8 (2.1–79.3)	47.5 (1.1–94.8)
TPO Ab (<6 IU/mL)	215.5 (3–820)	232 (20.5–373)

TSH, thyroid-stimulating hormone; TSAb, TSH receptor stimulating Ab; TBAb, TSH receptor blocking Ab; fT3, free triiodothyronine; fT4, free thyroxine; Tg Ab, anti-thyroglobulin Ab; TPO Ab, anti-thyroid peroxidase Ab.

Functional TSHR Antibody Specificity

Ten TBAb-Positive AITD Patients Tested in the Bio- and Binding Assays

All 30 TSAb-positive GD patients were negative for TSHR blocking Ab as tested in the TBAb bioassay. On the other hand, 10 patients with AITD (GD: $n = 6$, HT: $n = 4$) were exclusively positive for TBAb in the TBAb bioassay and were negative for TSAb in the TSAb bioassay. All 10 TBAb-positive/TSAb-negative serum samples tested positive in the 4 binding assays: Immulite, Kronus, Kryptor, and Dynex (Table 3).

Monoclonal Antibodies

Mixtures of 100% K1-70 (200 ng/mL), 80% K1-70 (160 ng/mL) + 20% M22 (4 ng/mL), 60% K1-70 (120 ng/mL) + 40% M22 (8 ng/mL), 40% K1-70 (80 ng/mL) + 60% M22 (12 ng/mL), 20% K1-70 (40 ng/mL) + 80% M22 (16 ng/mL), and 100% M22 (20 ng/mL) were 39, 80, 165 (positive), 262 (positive), 361 (positive), and 415 (positive) SRR%, respectively, for the TSAb bioassay, as presented in Figure 2a, while they were 82, 61, 24 (negative), –26 (negative), –77 (negative), and –95% (negative) inhibition, respectively, for the TBAb bioassay, as shown in Figure 2b. The TBAb bioassay showed 100% specificity when testing M22 stimulating Ab and K1-70 blocking Ab concurrently. Mixtures of 100% K1-70 (200 ng/mL), 80%

Fig. 1. Comparison of results for TSHR Ab binding immunoassays and a TSAb bioassay on undiluted and diluted (1:3 to 1:2,187) samples from 30 hyperthyroid patients with untreated GD. The bars indicate the number of positive samples for the TSAb bioassay, Kronus ELISA, Immulite, Kryptor, and Dynex ELISA, respectively. On undiluted samples, differences were noted between the bioassay and the Kryptor ($p = 0.063$) and Dynex ($p = 0.031$). Already

on the 1:3 dilution, marked differences were observed between the TSAb bioassay and the Kronus ELISA ($p = 0.031$), Immulite ($p = 0.016$), Kryptor ($p < 0.001$), and Dynex ELISA ($p < 0.001$). On both 1:9 and 1:27 dilutions, major differences were noted between the TSAb bioassay and all 4 binding assays (all $p < 0.001$). On the 1:81 dilution, differences were still observed between the TSAb bioassay and the Kronus ELISA, ($p = 0.039$) and Immulite ($p = 0.002$).

Table 2. TBII binding assay and TSAb/TBAb bioassay values in 40 undiluted serum samples from patients with autoimmune thyroid disease

	Untreated hyperthyroid patients with Graves' disease	Patients with Graves' disease or Hashimoto's thyroiditis on levothyroxine
<i>Patients, N</i>	30	10
Kronus Elisa (<1 IU/L)	10 (4.9; 14.8)	30 (25.8; 39.3)
Dynex Elisa (<2 U/L)	6.1 (3; 14.3)	45 (34; 51.5)
Immulite (<0.55 IU/L)	3.5 (1.5; 8.9)	36.3 (3.1; 40)
Kryptor (<1.8 IU/L)	4.9 (2.6; 9.5)	18.8 (16; 53.4)
TSAb bioassay (<140 SRR%)	367 (242; 472)	70 (53; 92)
TBAb bioassay (<40% inhibition)	-121 (-156; -75)	82 (68; 99)

TBII, thyroid-binding inhibitory immunoglobulins; TSAb, thyroid-stimulating hormone receptor stimulating Ab; TBAb, thyroid-stimulating hormone receptor blocking Ab; SRR%, specimen-to-reference ratio.

K1-70 (160 ng/mL) + 20% M22 (4 ng/mL), 60% K1-70 (120 ng/mL) + 40% M22 (8 ng/mL), 40% K1-70 (80 ng/mL) + 60% M22 (12 ng/mL), 20% K1-70 (40 ng/mL) + 80% M22 (16 ng/mL), and 100% M22 (20 ng/mL) all tested positive in both the Immulite (26.4, 20.2, 15.2, 10.5, 6.3, 2.00 IU/L) (Fig. 2c) and the Kronus assays (27.1, 23.3, 19.3, 12.0, 5.7, 2.2 IU/L) (Fig. 2d). Overall, the antibody mixture study demonstrated higher specificity of the bioassays over the binding assays.

Discussion

This original study compared for the first time the assay performance and dilution analysis of 6 immunoassays using well-characterized serum samples from clearly defined patients with AITD. This comparative immunoassay study encompassed both newly introduced automated binding assays, widely distributed ELISA assays, and FDA-cleared and/or validated cell-based bioassays. The

Fig. 2. Comparison of the TSAb/TBAb bioassays and 2 immunoassay results of mixtures of M22 and K1-70 MAb. **a** TSAb bioassay. The MAb mixtures are shown on the *x* axis and the percentage of specimen-to-reference ratio (SRR%) on the *y* axis. The dashed line indicates the cutoff at 140 SRR%. Mean $\pm$ SEM values are shown. **b** TBAb bioassay. The MAb mixtures are shown on the *x* axis and the percent inhibition on the *y* axis. Positive percent inhibition reflects blocking activity and negative percent inhibition reflects stimulating activity. The dashed line indicates the assay cutoff at 40% inhibition. Mean $\pm$ SEM values are shown. **c** Immulite assay. The MAb mixtures are shown on the *x* axis and international units per liter (IU/L) on the *y* axis. The dashed line indicates the assay cutoff at 0.55 IU/L. Mean $\pm$ SEM values are shown. **d** Kronus assay. The MAb mixtures are shown on the *x* axis and international units per liter (IU/L) on the *y* axis. The dashed line indicates the assay cutoff at 1 IU/L. Mean $\pm$ SEM values are shown.

Table 3. Binding assay and bioassay results for 10 TBAb-positive/TSAb-negative samples from patients with autoimmune thyroid disease (either Hashimoto's thyroiditis or Graves' disease)

Patients with AITD	TBAb bioassay (<40% inhibition)	Immulite (<0.55 IU/L)	Kronus Elisa (<1IU/L)	Kryptor (<1.8 IU/L)	Dynex Elisa (<2 U/L)	TSAb bioassay (<140 SRR%)
1	100	>40	>40	18.5	35.9	56
2	100	>40	>40	19.0	28.3	54
3	84	>40	38	18.5	41.1	45
4	98	34	39	16.5	39.1	36
5	83	0.784	32	1.9	2.1	78
6	69	38.8	27	14.4	49.8	95
7	64	38.5	25	41.5	51.3	105
8	78	33.2	26	55.5	53.0	91
9	81	2.03	28	52.7	48.9	89
10	59	3.47	19	56.8	52.0	61

AITD, autoimmune thyroid disease; TSAb, thyroid-stimulating hormone receptor stimulating Ab; TBAb, thyroid-stimulating hormone receptor blocking Ab; SRR%, specimen-to-reference ratio.

direct comparison demonstrated significant differences in the performance of the 6 assays. The most striking difference was the higher sensitivity of the cell-based TSAb bioassay, which remained positive both at the highest dilution of sera and at the lowest antibody concentration. Surprisingly, the performance was better in an ELISA assay, using the MAb M22 as a ligand to the TSHR, com-

pared to the latest generation of automated TSHR Ab binding assays.

All patients with AITD were carefully selected according to the diagnoses of GD and HT defined in the recently published guidelines of the American Thyroid Association (ATA) on hyperthyroidism management [18]. The serum samples from untreated patients with GD

were tested and reproduced at least 3 times in the TSAb bioassay. Marked and statistically significant differences were then noted in the undiluted patient serum samples between the TSAb bioassay and 2 out of 4 binding assays. A significant lower accuracy and sensitivity was observed, especially between the bioassay and the Kryptor and Dynex assays. Furthermore, the dilution analysis demonstrated marked differences between the bioassay and all 4 binding assays at high dilutions. In comparison, a dilution analysis of 20 untreated patients with GD selected from a prospective trial with antithyroid drug treatment demonstrated large and significant differences between the bioassay and the automated Cobas binding assay, with 250- to 800-fold differences of magnitude pertaining to the limit of detection, limit of quantitation, and half-maximal effective concentration, EC_{50} [14]. To the best of our knowledge, there are no other studies on dilution analysis that allow a direct comparison of results from multiple commonly used anti-TSHR assays.

The considerably higher performance of the bioassays could be clinically relevant in a number of circumstances, such as in patients with low serum TSHR Ab levels which may be observed in mild and/or subclinical thyroid dysfunction, in recent onset of GD, and in the euthyroid variant of orbitopathy. Furthermore, it has been reported that both children [19] and adults with euthyroid orbitopathy [10, 20, 21] can test positive for TSAb and negative for TBII. Greater assay sensitivity might also lead to improved follow-up during the medical management of patients with Graves' hyperthyroidism and timing of treatment cessation. Accurate measurement of these Ab at the end of the usual 1-year treatment period would be clinically useful in deciding whether or not to terminate treatment.

The second part of this work showed the exclusive capability of the bioassays to specifically identify TSHR Ab functionality. Indeed, only bioassays were able to exclusively differentiate between TSHR stimulating and blocking Ab activity. For example, 10 samples from well-defined patients with AITD were positive for TSHR blocking Ab and negative for TSHR stimulating Ab in the cell-based bioassays. However, all patients tested positive in the automated (Immulate and Kryptor) and ELISA (Kronus and Dynex) binding assays. Distinguishing between TSAb and TBAb is relevant for accurate diagnostic and therapeutic management of AITD, especially during the critical period of pregnancy and postpartum, during AITD progression, and during the medical management of patients with Graves' hyperthyroidism. Both a shift from stimulatory to blocking activity during pregnancy

[22] and spontaneous changes of TSHR Ab functionality [23] have been described. Within a large prospective trial in patients with GD performed at our institution, medical antithyroid treatment led to either a shift of Ab functionality and/or the appearance of both stimulating and blocking TSHR Ab in approximately 10% of the treated patients [24]. Also, the presence of blocking TSHR Ab had a significant impact on the probability of remission. Finally, blocking TSHR Ab caused hypothyroidism in patients with AITD, foremost in HT [25, 26].

In summary, the results obtained in the present study support both superior performance and exclusive functional Ab specificity of the cell-based bioassays compared to novel automated and widely distributed ELISA binding assays. These results have implications for the clinical management of patients with AITD.

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Statement of Ethics

This research complies with the guidelines for human studies.

Disclosure Statement

T.D., C.W., A.U., J.K., M.K., A.B., and B.D. have nothing to disclose. P.D.O. and G.J.K. consult for Quidel.

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