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Third generation radioimmunoassay (RIA) for TSH receptor autoantibodies (TRAb) – one step less, similar results?

Drittgenerations – Radioimmunoassay (RIA) für TSH-Rezeptor-Autoantikörper (TRAK) – ein Schritt weniger, gleiche Ergebnisse?

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ABSTRACT

Aim TSH-receptor (TSHR)-autoantibody (TRAb) is the serological hallmark of Graves' disease (GD). Recently, 3rd-generation radioimmunoassays (RIA) employing monoclonal TRAb such as M22 or T7 instead of TSH for the inhibition of human TRAb binding with solid-phase TSHR (coated tubes) have been introduced into laboratory routine.

Methods As current assays typically employ a consecutive incubation of patient serum and labelled monoclonal TRAb, automation of TRAb RIA is a challenge. Thus, the assay procedure using human TSHR-coated tubes and the mouse monoclonal TRAb T7 was modified by combining both steps. The novel one-step method was compared with its corresponding consecutive 3rd-generation RIA by investigating 304 individuals encompassing 102 patients with active GD (GD_a), 43 patients with GD after successful therapy (GD_t), 31 with Hashimoto's disease (HD), 28 with non-autoimmune thyroid diseases (NAITD) and 100 healthy subjects (HS).

Results With the new method, the incubation time was shortened by approximately one hour. Both 3rd-generation RIAs did not reveal a significantly different assay performance by comparing areas under the curve (AUC) with receiver operating characteristics curve analysis (AUC one-step: 0.94, AUC two-step: 0.96, $p > 0.05$, respectively). The two-step TRAb RIA demonstrated sensitivity and specificity values of 87.5 % and 96.2 %, respectively, whereas the one-step revealed 84.6 % and 96.2 %, respectively.

Conclusion One-step 3rd-generation RIA may be used for the reliable detection of TRAb. The shorter and easier assay design may improve its use and enable automation in routine nuclear medicine laboratories.

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ZUSAMMENFASSUNG

Zielsetzung TSH-Rezeptor (TSHR)-Autoantikörper (TRAb) sind die pathogenetische Ursache des Morbus Basedow (GD). Die Bestimmung von TRAb in der Routinediagnostik ist mit Radioimmunoassays (RIA) der dritten Generation möglich. Diese verwenden monoklonale TRAb wie M22 oder T7 anstatt TSH, um die Bindung von TRAb an Festphasen-immobilisierte TSHR (coated tubes) zu inhibieren. Herkömmliche Assays lassen Patientenserum und markierte monoklonale TRAb nacheinander inkubieren. Dies erschwert eine Automatisierung des Assays.

Methoden Der Assay mit humanen TSHR-coated tubes und monoklonalem TRAb T7 wurde modifiziert, indem die beiden Schritte kombiniert wurden. Die neue Einschrittmethode wurde mit ihrem korrespondierenden RIA der dritten Generation verglichen. Eingeschlossen wurden 304 Individuen, davon 102 Patienten mit derzeit aktivem Morbus Basedow (GD_a), 43 Patienten mit erfolgreich behandeltem Morbus Basedow (GD_t), 31 Patienten mit Hashimoto-Thyreoiditis (HD), 28 Patienten mit nichtautoimmuner Schilddrüsenerkrankung (NAITD) und 100 gesunde Individuen (HS).

Ergebnisse Die Ablaufzeit des Assays konnte um etwa 1 Stunde verringert werden. Die beiden Assays unterschieden sich nicht signifikant voneinander bezüglich des Vergleichs der Lachen unter den Kurven (AUC) in der ROC-Analyse (AUC Einschrittmethode: 0,94; AUC Zweischrittmethode: 0,96; $p > 0{,}05$). Der herkömmliche TRAb-RIA zeigte jeweils eine Sensitivität und Spezifität von 87,5 % and 96,2 %, während die Werte des Einschrittassays bei 84,6 % und 96,2 % lagen.

Schlussfolgerung Die Einschrittmethode des RIA der dritten Generation kann für die verlässliche Bestimmung von TRAb verwendet werden. Das kürzere und einfachere Assaydesign kann die Anwendbarkeit der Methode verbessern und ihre Automatisierung in der nuklearmedizinischen Routinediagnostik ermöglichen.

Introduction

Graves' disease (GD) is an autoimmune thyroid disease (AITD) with systemic consequences due to the elevated secretion of thyroid hormones caused by stimulating autoantibodies against the thyroid stimulating hormone receptor (TSHR). Graves' disease is a frequent AITD with an incidence of 20–30 cases in 100 000 individuals per annum in iodine-replete areas [27]. It affects up to 1 % of the population and is one of the most prevalent autoimmune illnesses [8, 10]. The detection of TSHR autoantibodies (TRAb) is recommended for the differential diagnosis of hyperthyroidism in all suspected patients according to the American guidelines [7]. The other thyroid gland-specific autoantibody against thyroid peroxidase is the serological tool for the diagnosis of hypothyroidism but is only recommended in cases with subclinical disease course.

Stimulating and inhibiting TRAb occur, however, cell-based bioassays are required to discriminate both TRAb types [5, 14]. The binding characteristics of these two TRAb types recognizing common overlapping epitopes of the TSH-binding site and their corresponding signal transduction via the TSHR are still poorly understood [17, 19, 32]. The recently established non-competitive TRAb bridge assay with similar assay performance to competitive binding TRAb assays has yet to demonstrate its discrimination in blocking and stimulating TRAb [6, 29].

Instead, since 1974, three generations of competitive binding assays have been introduced due to their practicability that simultaneously analyse both stimulating and blocking TRAb with improving assay performance [26, 32, 34]. These TRAb assays depend on patient's TRAb inhibition of labelled TSH binding to TSHR preparations in fluid phase (1st generation) or immobilized TSHR on solid phases (2nd and 3rd-generation assay designs) [4, 15, 24]. In particular, the use of the latter has resulted in a continuous improvement of the sensitivity [34]. These assays are available in different variants such as radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), electroluminescence assay, chemiluminescence assay, fluoroenzyme assay and others [2, 11, 13, 24, 28, 29]. With the introduction of the 2nd-generation TRAb RIA, this variant was also referred to as radioreceptor antibody assay [16]. The latest competitive assay generation encompasses the use of solid phase-immobilized TSHR and a monoclonal TRAb recognizing the TSH binding site thereof and replacing TSH as competitive reagent [22]. The use of human or porcine TSHR, immobilized onto solid phases such as 96-well plates (ELISA) or polystyrene tubes (RIA), did not appear to result in significantly different assay performance in this context [4, 23, 35]. The human stimulating monoclonal TRAb M22 was the first to be used in an ELISA to detect TRAb [25, 33]. It was later shown that other monoclonal TRAb, such as the murine T7, were not inferior to the former in similar assay designs [20].

Despite the more and more limited use of RIA with ^{125}I-labelled reagents in laboratory medicine as a whole, TRAb RIA is still appreciated in nuclear medicine laboratories due to its robust performance. However, the rising demand for laboratory services also necessitates higher throughput and thus automation in this area [2]. In order to facilitate 3rd-generation TRAb RIA automation, we developed a one-step incubation procedure for the detection of TRAb by employing the mouse monoclonal TRAb T7. The novel assay was compared to its two-step counterpart with consecutive incubation of patient serum and ^{125}I-labelled T7 and further evaluated in clinically defined patients and controls.

Patients and Methods

We analysed 204 serum samples of adult patients from the Thyroid Unit of the Department of Nuclear Medicine of the University Hospital Carl Gustav Carus and 100 samples of apparently healthy subjects (HS) (in.vent, Hennigsdorf, Berlin) as controls. Patients suffering from GD ($n = 145$) were divided into two subgroups of 104 active GD (GD_a) patients and 41 after successful therapy (GD_t).

▶ **Table 1** Demographic and serological data of patients and controls.

		GD_a	GD_t	NAITD	HT	HS
n (gesamt)		104	41	28	31	100
n (female)		91	37	19*	27	48*
age (median)		52	60*	64*	44*	26.5*
age (IQR)		23.5	22	21.5	23	13
TSH mU/L	< 0.3	61	9	0	3	nd
	0.3–4.0	26	20	0	19	nd
	> 4.0	17	12	28	9	nd
	median	0.07	1.64*	64.5*	2.19*	nd
	IQR	2.3325	5.32	21.5	3.4	nd
fT4 pmol/L	< 9.4	17	5	1	1	nd
	9.4–25.0	71	32	12	26	nd
	> 25.0	15	4	0	3	nd
	median	16.11	15.78	15.45	17.285	nd
	IQR	9.375	7.4	3.63	6.42	nd
fT3 pmol/L	< 3.4	10	4	1	3	nd
	3.4–7.2	57	35	12	27	nd
	> 7.2	36	2	0	0	nd
	median	6.01	4.71*	4.82	4.33*	nd
	IQR	4.19	1.43	1.11	0.91	nd
TgAb (U/mL)	< 50	46	22	22	7	nd
cut-off: 50 U/mL	> 50	58	19	6	24	nd
	median	77.5	29	20*	181	nd
	IQR	357.25	210	10.25	282	nd
TPOAb (units/mL)	< 50	23	14	15	3	nd
cut-off: 50 U/mL	> 50	81	27	13	28	nd
	median	199.5	161	20*	280	nd
	IQR	451.5	483	189	403.5	nd

* $p < 0.05$ regarding the comparison of Graves' disease patients with control cohorts.

Twenty-eight patients suffering from non-autoimmune thyroid diseases (NAITD) and 31 from Hashimoto's disease (HD) were enrolled as disease controls (▶ **Table 1**). Levels of TSH, triiodothyronine (T3), thyroxine (T4), thyroglobulin antibodies (TgAb) and thyroid peroxidase antibodies (TPOAb) were determined by electro chemiluminescent immunoassay (ECLIA) on the cobas modular analytics system (Roche Diagnostics).

The diagnosis of GD was based on multiple factors such as clinical symptoms of hyperthyroidism, supressed TSH and elevated thyroid hormone levels, thyroid ultrasound and thyroid scintigraphy.

The patients were at various disease stages (e. g., some were on thyroid medication, had radioiodine treatment or thyroidectomy recently). Only patients with normalized thyroid hormone levels without ongoing medication of thyreostatics were recruited into the GD_t group. This was the case either after treatment of thyreostatics or after definitive therapy (radioiodine therapy or thyroidectomy).

Hashimoto's disease was diagnosed by repeated clinical examination of patients resulting in reasonable suspicion. Ultimate development of hypothyroidism, interrupted by periods of borderline thyrotoxicosis was observed as well as elevated levels of TPOAb and/or TgAb, reduced parenchyma and hypoechoic lesions in ultrasound and lymphocytic infiltrates in fine needle aspiration cytology. Of course, some uncertainty remained, especially in patients with positive TRAb levels.

The group of NAITD was recruited based on clinically overt hyperthyroidism, suppressed TSH, elevated thyroid hormone levels, negative TRAb by the two-step assay and confirmation of diffuse or nodular goitre by ultrasound and scintigraphy. Thirteen patients demonstrated positive levels of TPOAb, one even had positive TRAb as well. However, due their clinical history with

repeated examinations over an extended period of time under the supervision of a senior consultant in Nuclear Medicine, the diagnosis was confirmed and all patients were not diagnosed with GD or HT. All patients were at various stages of their diseases (e. g., some were on thyroid medication, had radioiodine treatment or thyroidectomy recently).

The study was approved by the ethics committee of the Faculty of Medicine of the Technical University Dresden (ethical permit number: EK 56 022 014). This study was conducted in accordance with the principles of the Declaration of Helsinki (World Medical Association Declaration of Helsinki 1989).

Thyroid autoantibody and hormone evaluation

Levels of TRAb were assessed by a commercial 3rd-generation RIA with consecutive incubation of human serum and ^{125}I-labelled monoclonal TRAb T7 as recommended by the manufacturer (Medipan, Dahlewitz, Germany). Briefly, 50 µL of start buffer and 100 µL of neat human serum were incubated in TSHR coated tubes at room temperature (RT) for 2 hours while shaking. In tests with different incubation times from 30 minutes to 4 hours, we found that after a period of 2 hours the maximum binding was reached. Subsequent to two washing steps, ^{125}I-labelled monoclonal TRAb T7 was incubated at RT for 1 hour while shaking. In the absence of patient TRAb, ^{125}I-labelled T7 binds to the immobilized TSHR. Alternatively, the more TRAb is present in the sample, the less ^{125}I-T7 is bound. After two additional washing steps, bound patient TRAb was detected by its ability to inhibit the binding of ^{125}I-labelled T7 to the receptor coated tubes during the second incubation.

Autoantibodies to thyroglobulin and thyroid peroxidase were determined by commercial assays using the cut-offs recommended by the manufacturer (Thermo Fisher, Berlin, Germany).

Thyroid hormones were analysed by commercial assays in accordance with the recommendations of the manufacturers.

Third generation one-step TRAb RIA

The mouse monoclonal TRAb T7 recognizing the TSH binding site of the TSHR was employed for the detection of TRAb in human sera by a novel 3rd-generation RIA technique. Human TSHR was immobilized to polystyrene tubes by a mouse monoclonal TRAb not interfering with the TSH-binding site as reported elsewhere [20]. In contrast to the current two-step TRAb RIA procedure, 100 µL of neat patient serum or calibrators were simultaneously incubated with 50 µL of start buffer and 100 µL of ^{125}I-labelled T7 in the TSHR coated tubes at RT for 2 hours while shaking. Thus, human TRAb concurrently competed with labelled monoclonal T7 for the binding to the immobilized TSHR. Prior to the measurement of bound radioactivity, the tubes were washed with 1 mL deionized or distilled water and residual liquid was thoroughly removed. The TRAb concentrations of samples were directly read off in IU/l against radioactive signals in counts per minute or respective binding rate values (B/B0) obtained by a γ counter (Berthold Technologies, Bad Wildbad, Germany). ▶ **Table 4** summarizes the main differences of both detection methods.

Statistical analysis

Statistical analysis was performed using R and MedCalc (MedCalc software, Belgium). Prevalence comparison between groups was done by two-tailed Fisher's exact test and Kruskal-Wallis test with a post-hoc analysis according to Conover [3]. Method comparison was performed by the Passing-Bablok regression model, a linear regression procedure with no special assumptions regarding the distribution of the samples and the measurement errors. The result of the analysis does not depend on the assignment of the TRAb values to X and Y. The slope and intercept are calculated with their 95 % confidence interval (CI). CUSUM test for linearity was used to examine the applicability of the Passing-Bablok method, therefore evaluating how well a linear model fits the data.

Results of both TRAb tests were compared by McNemar's test and by Cohen's kappa test. Furthermore, assay performance data such as specificity, sensitivity, and positive and negative likelihood ratios as well as area under the curve (AUC) were determined by receiver operating characteristic (ROC) curve analysist. Significance was defined as $p < 0.05$.

Results

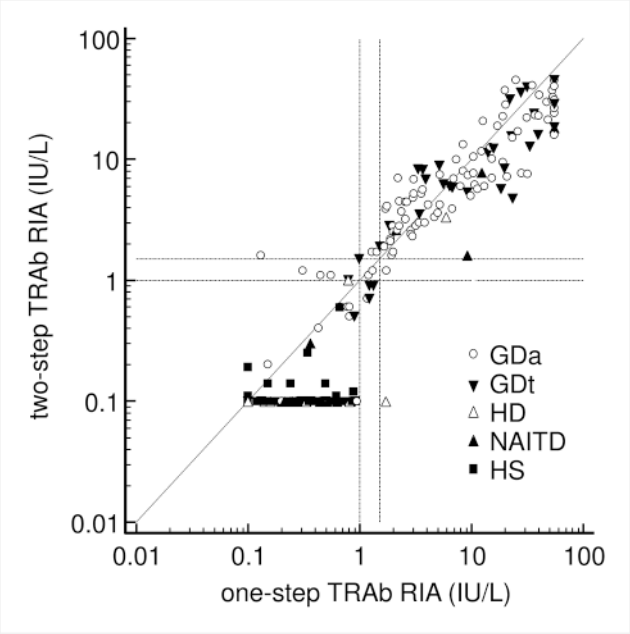
Demographic and thyroid laboratory characteristics of patients and controls

The study cohorts were matched by gender except for the group of HS that showed a significantly higher percentage of females ($p < 0.0001$). The cohorts were not matched by age according to Kruskal Wallis test ($p < 0.0001$). However, posthoc analysis did not show a significant difference between patients with GD_a and NAITD ($p > 0.05$). Patients with GD demonstrated significantly lower TSH and higher T3 levels than patients with HD ($p < 0.0001$, respectively). Regarding thyroid autoantibodies, both groups of autoimmune diseases GD and HD showed significantly higher levels of TgAb ($p = 0.0064$, $p = 0.0051$) and TPOAb ($p = 0.0001$, $p = 0.0051$) than the NAITD group as expected. Demographic and laboratory data are given in ▶ **Table 1**.

Assay performance of the one-step TRAb

Patient serum and labelled ^{125}I-labelled monoclonal TRAb T7 volumes were transferred to a reaction environment with concurrent incubation of all reagents including the starting buffer. Kinetic measurements of the labelled T7 TRAb revealed saturation of binding to the immobilized human TSHR after 90 min. In order to establish a robust assay design and to achieve an equilibrium of human and mouse monoclonal TRAb binding, a two-hour simultaneous incubation of all reagents was chosen. The use of deionized water for the washing step after incubation was sufficient to accomplish the separation of bound and free TRAb on the surface of the tubes.

The assay was calibrated against the WHO international standard for thyroid-stimulating antibody (NIBSC code 08/204). Interassay coefficients of variation (CV) were determined in accordance with CLSI protocol EP15-A2 using three different lots at



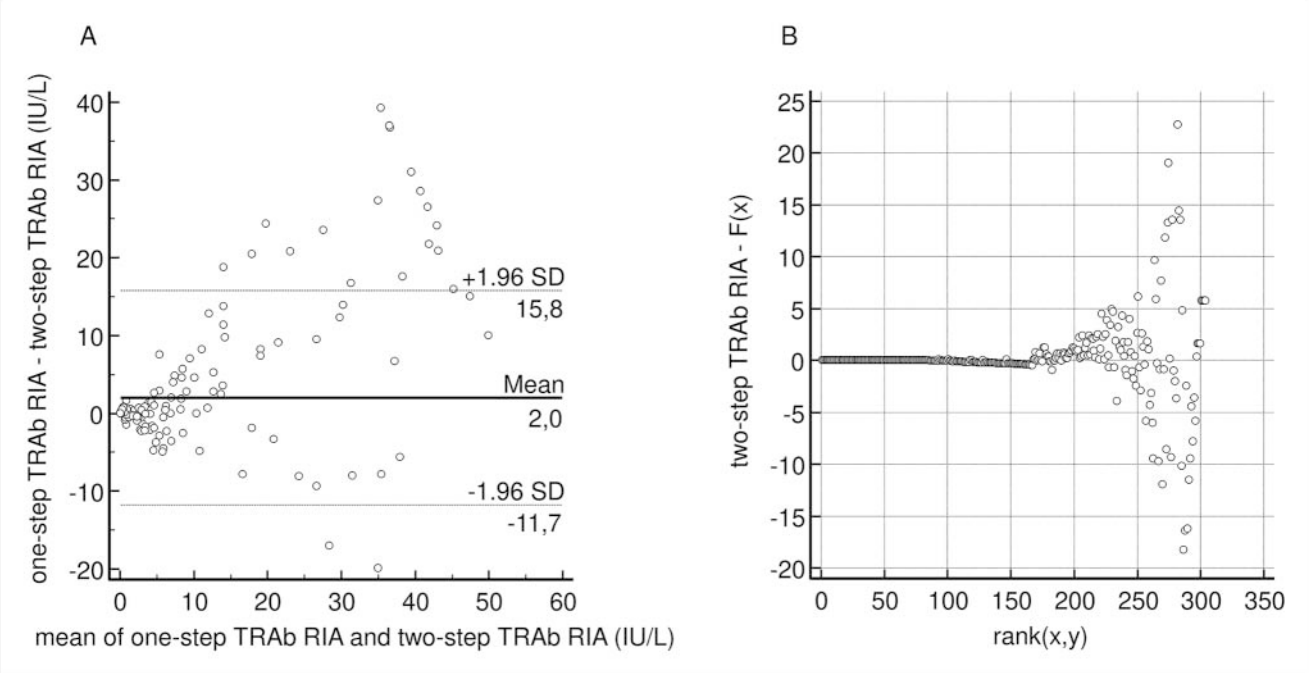
▶ **Fig. 1** Comparison of TSH-receptor autoantibody (TRAb) levels determined by one-step and two-step TRAb radioimmunoassays (RIAs) using the mouse monoclonal T7 as TSH receptor-binding molecule for competing with TRAb in 104 patients with active Graves' disease (GD_a), 41 with GD after successful therapy (GD_t), 31 with Hashimoto's disease (HD), 28 with non-autoimmune thyroid diseases (NAITD), and 100 healthy subjects (HS). The diagonal line demonstrates the line of equality. The vertical and horizontal lines represent cut-off values for equivocal (1 IU/L) and positive values (1.5 IU/L) for both assay variants, respectively.

19.5 % for a serum with a TRAb value of 0.8 IU/L and 9.5 % for sera with TRAb values of 2.8 IU/L and 14.6 IU/L, respectively.

The functional assay sensitivity (fas) of the mouse mAb T7-based one-step TRAb RIA was determined at the TRAb concentration of 0.8 IU/L with an inter-assay CV of 20 %. Thus, eight-fold determinations for eight TRAb-positive sera with different TRAb levels were obtained on five different days. For interference experiments, TRAb containing sera were spiked with human haemoglobin (1.0–10.0 g/L), triglycerides as 20 % emulsion of soybean oil (1.0–5.0 g/L) and unconjugated bilirubin (1.0–150.0 mg/L) (Sigma Co, respectively). The indicated reagent concentrations did not interfere with the detection of TRAb by the one-step assay.

Regression analysis of one-step and two-step TRAb RIAs

For all 304 sera, we determined TRAb levels by the two-step method with consecutive incubation of serum and ^{125}I-labelled monoclonal TRAb and by the novel one-step assay (▶ **Fig. 1**). Comparison of obtained TRAb values revealed the following regression equation by Passing-Bablok regression analysis: $y = 0.69 \times - 0.03$ (95 % confidence intervals [CI] for slope 0.63–0.74 and intercept 0.03–0.04). There were a proportional as well as a constant difference between both methods as the 95 % CI for slope and intercept did not cover the values 1 and 0, respectively. The corresponding residual plot and the Bland Altman plot are shown in ▶ **Fig. 2**. Accordingly, TRAb values of up to 15 IU/L demonstrated differences within the 1.96 standard deviation area. There was a significant deviation from linearity by CUSUM test ($p < 0.01$, respectively). The residual plot showed no clear tendency of TRAb value differences.



▶ **Fig. 2** Bland-Altman and corresponding residual plots for the comparison of one-step and two step TSH-receptor autoantibody (TRAb) radioimmunoassays (RIAs).

▶ **Table 2** comparison of TRAb positivity, diagnostic sensitivity, specificity, positive likelihood ratio (+LR) and negative likelihood ratio (–LR).

	number positive/total number								
TRAb RIA [cut-off]	**GD_a**	**GD_t**	**NAITD**	**HT**	**HS**	**sensitivity (%)**	**specificity (%)**	**+LR**	**–LR**
one step [1.5 IU/l]*	83/104	26/41	2/28	4/31	0/100	79.81 %	96.23 %	21.15	0.21
two step [1.5 IU/l]*	86/104	27/41	1/28	4/31	0/100	82.69 %	96.86 %	26.30	0.18
one step [1.0 IU/l]**	88/104	29/41	2/28	4/31	0/100	84.62 %	96.23 %	22.42	0.16
two step [1.0 IU/l]**	91/104	28/41	2/28	4/31	0/100	87.50 %	96.23 %	23.19	0.13

* cut-off values recommended by the manufacturer.
** cut-off values obtained by receiver operating characteristics curve analysis.

Analysis of TRAb by the one-step assay in patients and controls

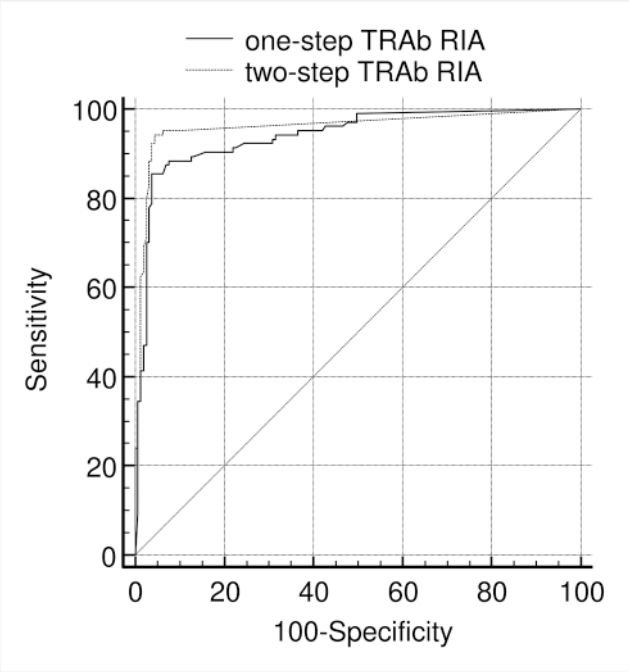
Patients with GD_a and GD_t demonstrated significantly higher TRAb levels than patients of all other disease groups as well as HS (Kruskal Wallis test with posthoc analysis, $p < 0.05$, respectively). Receiver-operating characteristics (ROC) curve analysis revealed 1.0 IU/L as ideal cut-off for the discrimination of positive and negative samples using the TRAb levels of 104 patients with GDa as disease variable and TRAb levels of 31 with HD, 28 with NAITD, and 100 HS as control variables. For the sake of assay robustness, values between 1.0 and 1.5 IU/L were considered equivocal. Corresponding assay performance characteristics such as sensitivity, specificity, positive likelihood ratio and negative likelihood ratio are given in ▶ **Table 2**. Using a cut-off of 1.0 IU/L, the one-step TRAb RIA demonstrated a sensitivity of 84.6 % (95 % CI 77.3 % – 91.7 %) and a specificity of 96.2 % (95 % CI 92.0 % – 98.6 %). With a cut-off of 1.5 IU/L, the sensitivity decreased to 79.8 % (95 % CI 69.7 %–86.2 %) with unchanged specificity.

Comparison of one-step and two-step TRAb RIAs

The two-step TRAb RIA revealed a higher sensitivity of 82.7 % with a comparable specificity of 96.9 % at a cut-off of 1.5 IU/L in contrast to the one-step assay. Though, McNemar's test did not reveal significant differences in qualitative results of both TRAb assay variants ($p > 0.05$). ▶ **Table 3** shows a detailed comparison of contingency tables by patient cohorts.

In total, agreement between the two assays was 98 % and shows a Cohen's kappa of 0.944. The agreement in GD_a and GD_t was 96 % for both assays and reached a Cohen's kappa of 0.867. The TRAb comparison of HD and HS showed perfect agreement, respectively, whereas both NAITD and GD_t cohorts showed one case of disagreement each. Nonetheless, these differences were not significant (McNemar's test, $p > 0.05$).

With respect to the quantitative comparison between the single-step and two-step TRAb RIAs, the areas under the curve (AUC) did also not show any significant differences by ROC curve analysis ($p = 0.1391$; ▶ **Fig. 3**).



▶ **Fig. 3** Comparison of receiver operating characteristic (ROC) curve analysis of one-step and two-step TSH-receptor autoantibody (TRAb) radioimmunoassays (RIAs) using the TRAb levels of 104 patients with active Graves' disease as disease variable and TRAb levels of 31 with Hashimoto's disease (HD), 28 with non-autoimmune thyroid diseases (NAITD), and 100 healthy subjects (HS). as control variable. The following areas under the curve (AUC) were obtained: one-step TRAb RIA: AUC = 0.941; 95 % CI: 0.906–0.966; two-step TRAb RIA: AUC = 0.960; 95 % CI: 0.928–0.980.

▶ **Table 3** contingency tables for comparison of qualitative TRAb results.

					two-step (> 1.5 IU/l)	
GD_a (n = 104)					pos	neg
				pos	82	1
	Cohens Kappa	0.84	one-step (> 1.5 IU/l)	neg	4	17
	p exact McNemars test	0.37				
GD_t (n = 41)					pos	neg
				pos	26	0
	Cohens Kappa	0.90	one-step (> 1.5 IU/l)	neg	1	14
	p exact McNemars test	1.00				
NAITD (n = 28)					pos	neg
				pos	1	1
	Cohens Kappa	0.65	one-step (> 1.5 IU/l)	neg	0	26
	p exact McNemars test	1.00				
HT (n = 31)					pos	neg
				pos	4	0
	Cohens Kappa	1.00	one-step (> 1.5 IU/l)	neg	0	27
	p exact McNemars test	na				
HS (n = 100)					pos	neg
				pos	0	0
	Cohens Kappa	na	one-step (> 1.5 IU/l)	neg	0	100
	p exact McNemars test	na				

▶ **Table 4** differences of one-step and two-step TRAb detection methods.

two step	one step
consecutive binding of human TRAb and labelled monoclonal TRAb to solid-phase immobilized TSHR	simultaneous binding of human TRAb and labelled monoclonal TRAb to solid-phase immobilized TSHR
two incubation steps (2 h + 1 h)	one incubation step (2 h)
two wash steps	one wash step
wash buffer required	no wash buffer (distilled or deionized water)
not adequately adaptable to current RIA processor automation	adaptable to current RIA processor automation

Discussion

For the assessment of TRAb as a pathognomonic marker for the differential diagnosis of hyperthyroidism, several assay techniques have been developed in the past 46 years [5, 32]. TSHR-binding competitive assays detect TSHR-binding inhibitory immunoglobulin which is commonly referred to as TRAb [32]. These TRAb assays do not discriminate stimulating and blocking TRAb and even the new bridge immunoassay for the detection of TRAb does not seem to enable this discrimination [1, 5]. Notwithstanding, three generations of TSHR-binding competitive assays have evolved to meet the clinical need for efficient and reliable TRAb analysis [34]. In this context, automation of TRAb RIAs has been hampered by the lack of appropriate instrumentation or inflexible assay design. The latter point was addressed in this study by developing a one-step 3rd-generation TRAb RIA with the mouse monoclonal TRAb T7 as inhibiting reagent.

The one-step TRAb RIA demonstrated equal assay performance to the two-step RIA. Statistical analysis did not reveal significant differences in qualitative as well as quantitative TRAb values. Consequently, simultaneous incubation of autoimmune TRAb of patients and the ^{125}I-labelled monoclonal TRAb T7 appears not to have a significant effect on TRAb assessment.

Nevertheless, regression analysis did not reveal perfect consistency due to significant differences in the slope and intercept.

This may point to binding differences in the one-step and two-step reaction environments.

When a cohort of 104 patients with GD_a was examined, the sensitivity of the new one-step method showed a 3 % reduction to 84.6 % with similar specificity compared to the two-step method. For assay performance evaluation, patients with GD_t were omitted due to the unpredictable effect of therapy. Nevertheless, in this cohort, the positivity rate of the one-step TRAb RIA was almost identical to the one of the two-step assay (26/41 versus 27/41).

The slightly reduced sensitivity of the one-step TRAb in our study may indicate an inferior binding efficacy for weakly TRAb-positive sera to compete with labelled T7 binding in the selected assay design. As a fact, the three GD_a patients with discrepant TRAb values showed low TRAb levels by the two-step TRAb RIA (< 2 IU/L). A similar phenomenon was reported by comparison of a 2nd generation TRAb assay using ^{125}I-labelled bovine TSH for the inhibition of patient TRAb to solid-phase immobilized human TSHR in a simultaneous reaction environment with its corresponding consecutive assay [9, 21] They concluded that the one-step TRAb assay detected particularly biologically active TRAb in patients with low TRAb levels. In contrast, the consecutive TRAb assay presumably also identified low affinity TRAb that lack thyroid stimulatory activity and did not reflect clinical symptoms.

Four HD patients out of 31 (12.9 %) demonstrated elevated TRAb by both assays. Two had low TSH levels (< 0.3 mU/L), however, they received thyroid hormone treatment. In previous studies the percentage of TRAb-positive patients with HD varied greatly and reached up to 10 % by using a 2nd-generation TRAb assay [12, 31] and even up to 50 % by a 3rd-generation assay [20, 36]. This may be due to blocking or apoptotic TRAb occurring in HD which can be picked up by the competitive assay design [5, 14, 18]. False-positive TRAb levels by a 3rd-generation assay were also reported in neonates which was, though, referred to effects of the sample matrix [30].

One patient with NAITD demonstrated moderately elevated levels of TRAb in both assays (two-step: 3.3 IU/l, one-step: 5.9 IU/l), which would point to the presence of GD. However, this aged patient receiving thyroid hormone replacement has a long disease history without autoimmune signs. Another patient with NAITD demonstrated slightly elevated levels of TRAb in the one-step (1.7 IU/l), but not in the two-step assay, which would also raise the question of autoimmune disease. However, the patient shows normal thyroid hormone levels and no other symptoms of GD.

Altogether, the one-step TRAb RIA may be used for analyzing TRAb for the serological diagnosis of GD instead of two-step assay designs. The one-step method reduced the assay time by 1 hour and, therefore, made it more applicable in routine nuclear medicine department laboratories. Though the main benefit is the simple assay design of a single incubation step which allows running the novel 3rd-generation TRAb RIA on currently available automated RIA processors.

Conflict of Interest

Grit Zarske and Dirk Roggenbuck are from Medipan GmbH, Berlin/Dahlewitz, Germany, and Medipan is a commercial manufacturer of TRAb assays including the presented TRAb assays here.

References

[1] Allelein S, Diana T, Ehlers M et al. Comparison of a Bridge Immunoassay with Two Bioassays for Thyrotropin Receptor Antibody Detection and Differentiation. Horm Metab Res 2019; 51 (6): 341–346

[2] Chauvet J, Leven C, Thuillier P et al. Comparison of the New, Rapid, and Fully Automated Kryptor TSH receptor Antibodies Assay (B.R.A.H.M.S.) with the Radioimmunological Assay (B.R.A.H.M.S.). Clin Lab 2019; 65 (11). doi:10.7754/Clin.Lab.2019.190124.

[3] Conover WJ. Practical nonparametric statistics. 1998; 3rd edition

[4] Costagliola S, Morgenthaler NG, Hoermann R et al. Second generation assay for thyrotropin receptor antibodies has superior diagnostic sensitivity for Graves' disease. J Clin Endocrinol Metab 1999; 84 (1): 90–97

[5] Diana T, Wuster C, Kanitz M et al. Highly variable sensitivity of five binding and two bio-assays for TSH-receptor antibodies. J Endocrinol Invest 2016; 39 (10): 1159–1165

[6] Frank CU, Braeth S, Dietrich JW et al. Bridge Technology with TSH Receptor Chimera for Sensitive Direct Detection of TSH Receptor Antibodies Causing Graves' Disease: Analytical and Clinical Evaluation. Horm Metab Res 2015; 47 (12): 880–888

[7] Garber JR, Cobin RH, Gharib H et al. Clinical practice guidelines for hypothyroidism in adults: cosponsored by the American Association of Clinical Endocrinologists and the American Thyroid Association. Thyroid 2012; 22 (12): 1200–1235

[8] Hayter SM, Cook MC. Updated assessment of the prevalence, spectrum and case definition of autoimmune disease. Autoimmun Rev 2012; 11 (10): 754–765

[9] Ishihara T, Saiki Y, Ikekubo K et al. Evaluation of a 1 step TRAb assay for the detection of high-affinity components to hTSHR: evidences indicating superiority of the assay in the lower TRAb range. Endocr J 2006; 53 (1): 147–155

[10] Jacobson DL, Gange SJ, Rose NR et al. Epidemiology and estimated population burden of selected autoimmune diseases in the United States. Clin Immunol Immunopathol 1997; 84 (3): 223–243

[11] Kakinuma A, Morimoto I, Kuroda T et al. Comparison of recombinant human thyrotropin receptors versus porcine thyrotropin receptors in the thyrotropin binding inhibition assay for thyrotropin receptor autoantibodies. Thyroid 1999; 9 (9): 849–855

[12] Kamijo K. TSH-receptor antibody measurement in patients with various thyrotoxicosis and Hashimoto's thyroiditis: a comparison of two two-step assays, coated plate ELISA using porcine TSH-receptor and coated tube radioassay using human recombinant TSH-receptor. Endocr J 2003; 50 (1): 113–116

[13] Kim JJ, Jeong SH, Kim B et al. Analytical and clinical performance of newly developed immunoassay for detecting thyroid-stimulating immunoglobulin, the Immulite TSI assay. Scand J Clin Lab Invest 2019; 79 (6): 443–448

[14] Lytton SD, Schluter A, Banga PJ. Functional diagnostics for thyrotropin hormone receptor autoantibodies: bioassays prevail over binding assays. Front Biosci (Landmark Ed) 2018; 23: 2028–2043

[15] Massart C, Orgiazzi J, Maugendre D. Clinical validity of a new commercial method for detection of TSH-receptor binding antibodies in sera from patients with Graves' disease treated with antithyroid drugs. Clin Chim Acta 2001; 304 (1): 39–47

[16] Meller J, Jauho A, Hufner M et al. Disseminated thyroid autonomy or Graves' disease: reevaluation by a second generation TSH receptor antibody assay. Thyroid 2000; 10 (12): 1073–1079

[17] Morgenthaler NG, Ho SC, Minich WB. Stimulating and blocking thyroid-stimulating hormone (TSH) receptor autoantibodies from patients with Graves' disease and autoimmune hypothyroidism have very similar concentration, TSH receptor affinity, and binding sites. J Clin Endocrinol Metab 2007; 92 (3): 1058–1065

[18] Morshed SA, Davies TF. Graves' Disease Mechanisms: The Role of Stimulating, Blocking, and Cleavage Region TSH Receptor Antibodies. Horm Metab Res 2015; 47 (10): 727–734

[19] Rapoport B, Chazenbalk GD, Jaume JC et al. The thyrotropin (TSH) receptor: interaction with TSH and autoantibodies. Endocr Rev 1998; 19 (6): 673–716

[20] Roggenbuck JJ, Veiczi M, Conrad K et al. A novel third-generation TSH receptor antibody (TRAb) enzyme-linked immunosorbent assay based on a murine monoclonal TSH receptor-binding antibody. Immunol Res 2018; 66 (6): 768–776

[21] Saiki Y, Ishihara T, Ikekubo K et al. Differences in TSH receptor binding and thyroid-stimulating properties between TSH and Graves' IgG. Slowly-acting TSH receptor antibody moieties in Graves' sera affect assay data. Endocr J 2005; 52 (1): 45–55

[22] Sanders J, Evans M, Premawardhana LD et al. Human monoclonal thyroid stimulating autoantibody. Lancet 2003; 362: 126–128

[23] Sanders J, Oda Y, Roberts S et al. The interaction of TSH receptor autoantibodies with 125I-labelled TSH receptor. J Clin Endocrinol Metab 1999; 84 (10): 3797–3802

[24] Shewring G, Smith BR. An improved radioreceptor assay for TSH receptor antibodies. Clin Endocrinol (Oxf) 1982; 17 (4): 409–417

[25] Smith BR, Bolton J, Young S et al. A new assay for thyrotropin receptor autoantibodies. Thyroid 2004; 14 (10): 830–835

[26] Smith BR, Hall R. Thyroid-stimulating immunoglobulins in Graves' disease. Lancet 1974; 2: 427–431

[27] Smith TJ, Hegedus L. Graves' Disease. N Engl J Med 2016; 375 (16): 1552–1565

[28] Struja T, Jutzi R, Imahorn N et al. Comparison of Five TSH-Receptor Antibody Assays in Graves' disease: results from an observational pilot study. BMC Endocr Disord 2019; 19 (1): 38

[29] Villalta D, D'Aurizio F, Da Re M et al. Diagnostic accuracy of a new fluoroenzyme immunoassay for the detection of TSH receptor autoantibodies in Graves' disease. Auto Immun Highlights 2018; 9 (1): 3

[30] Wada M, Kita M, Kawasaki K et al. False-positive TSH receptor antibody – a pitfall of third-generation TSH receptor antibody measurements in neonates. Endocr J 2018; 65 (5): 587–592

[31] Zophel K, Gruning T, Roggenbuck D et al. On specificity of 2nd generation TSH receptor autoantibody measurements. Clin Lab 2008; 54 (7): 243–249

[32] Zophel K, Roggenbuck D, Schott M. Clinical review about TRAb assay's history. Autoimmun Rev 2010; 9 (10): 695–700

[33] Zophel K, Roggenbuck D, von Landenberg P et al. TSH receptor antibody (TRAb) assays based on the human monoclonal autoantibody M22 are more sensitive than bovine TSH based assays. Horm Metab Res 2010; 42 (1): 65–69

[34] Zophel K, Roggenbuck D, Wunderlich G et al. Continuously increasing sensitivity over three generations of TSH receptor autoantibody assays. Horm Metab Res 2010; 42 (12): 900–902

[35] Zophel K, von Landenberg P, Roggenbuck D et al. Are porcine and human TSH receptor antibody measurements comparable? Clin Lab 2008; 54 (1): 1–8

[36] Zophel K, Wunderlich G, Kotzerke J et al. M22 based (manual) ELISA for TSH-receptor antibody (TRAb) measurement is more sensitive than 2nd generation TRAb assays. Clin Chim Acta 2009; 403 (1): 266