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ORIGINAL ARTICLE

Accuracy of receptor-based methods for detection of thyrotropin-receptor autoantibodies: a new automated third-generation immunoassay shows higher analytical and clinical sensitivity for the differential diagnosis of hyperthyroidism

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Abstract *Purpose*: Specific autoantibodies acting as TSH receptor agonists (TRAb) are responsible for Graves' disease (GD). In the last 30 years three generations of assay methods for the detection of TRAb have become available. The aim of this multicentre study was to evaluate the analytical sensitivity, precision and diagnostic accuracy of TRAb measurement using a new automated assay in comparison with a second-generation standard method. *Methods*: Serum samples from patients with GD (*n*=82), autoimmune thyroiditis (AIT, *n*=57) or hyperthyroidism (HT, n=292), from 106 healthy subjects and from 57 patients with infectious diseases were analysed using a third-generation TRAb immunoassay (anti-TSHR, RAD 120; Radim, Italy) based on the human monoclonal TSH receptor antibody M22. *Results*: Using a cut-off value of 1.25 mIU/l, established by ROC curve analysis, 80/82 GD patients (97.5%), 68/292 HT patients (23.2%), and 6/57 AIT patients (10.5%) were TRAb-positive with the M22-based automated assay. The percentages of TRAb positivity were lower in the same patients when the measurements were done with the second-generation method (95.1%, 18.9%, 7.0%, respectively). *Conclusion*: The M22-based automated immunoassay shows high functional sensitivity (0.4 mIU/l) and high diagnostic specificity, is more sensitive than the standard second-generation method and is less time-consuming and labour-intensive, and is therefore the up-to-date technology for TRAb detection in clinical practice.

Keywords TSHR antibodies · Automated TRAb immunoassay · Graves' disease · Hyperthyroidism · Sensitivity · Specificity

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Introduction

The thyrotropin receptor (TSHR) is the major autoantigen in autoimmune hyperthyroidism (HT) and specific autoantibodies acting as TSHR agonists (TRAb) are responsible for clinical manifestations and are a diagnostic hallmark of Graves' disease (GD) [1]. Measurement of TRAb plays a decisive role in the differentiation of HT into its nosological types, which is important for therapeutic and prognostic purposes [2].

After the discovery by Adams and Purves of TRAb (initially called long-acting thyroid stimulator – LATS) as a cause of HT [3] and identification of LATS as an immunoglobulin [4], until the early 1970s the only available methods for detection of TRAb were in vivo bioassays based on the original principle of McKenzie [5].

Following early seminal experiments [6, 7], Rees Smith and Hall in the early 1980s were the first to describe a competitive receptor immunoassay with inhibition of TSH binding on the TSHR [8, 9]. Further modifications of the analytical procedure and the commercial availability of reagents have made this assay the method of choice for TRAb measurement in most clinical laboratories [10, 11]. These methods, based on the principle of the inhibition of ^{125}I-TSH (radioreceptor assay) or enzyme-labelled TSH binding (enzyme-receptor assay) are referred to as first-generation immunoassays.

Despite a high specificity (nearly 100%), these assays show a low diagnostic sensitivity, ranging from 52% to 94% (with a mean of 77.5%) in the most relevant literature reports of the last 20 years [10, 12–20] (Table 1). As a consequence, a significant proportion (6–48%; mean 22.5%) of GD patients with clinical HT were reported to be TRAb 'negative' by first-generation methods. These results may depend on differences in the type of patients studied (untreated or treated GD), the methodologies used (commercial or in-house), the source of TSHR (recombinant human or purified porcine) and the assay procedure (times of incubation, positivity thresholds, reference values). In general, methods using recombinant human TSHR show higher diagnostic sensitivity.

To increase the sensitivity of the TRAb assay, second-generation immunoassays that use monoclonal antibodies (mAb), human or porcine TSHR immobilized on a plastic surface and bovine TSH labelled with ^{125}I, or acridinium ester, or with biotin-streptavidin-peroxidase have become available [15, 21]. Several studies have shown that the clinical sensitivity of these assays increases to a mean of 95.9% (range 72.6–100%; Table 1) with a small decrease in specificity (97.9%, range 91.4–100%) [15, 17–19, 22–30]. The recombinant human TSHR-based second-generation assay, in combination with labelled bovine TSH, is now considered the gold standard with the highest diagnostic accuracy.

Recently, a new method for measuring TRAb has been described by Rees Smith [31], in which autoantibodies inhibit the binding of a human thyroid-stimulating mAb M22 [32] labelled with biotin to TSHR-coated ELISA plate wells. The method is called the manual third-generation TRAb assay. However, the diagnostic accuracy of this manual ELISA is similar to that of the second-generation TRAb assay methods [24, 26, 28–31] (Table 1). The main limitation of these assays is their poor analytical sensitivity and imprecision due to the manual nature of the procedure.

To overcome these limitations, the first fully automated electrochemiluminescence immunoassay based on the M22 procedure was developed 3 years ago and has become commercially available [33]. This method is called the automated third-generation TRAb assay. Five recent studies using the unique commercial technology (Roche Diagnostics) have demonstrated that the diagnostic sensitivity (97.7%) and specificity (99.5%) are higher than those of the second-generation TRAb assays (94.5% and 98.4%, respectively; Table 1) [25, 26, 33–35].

More recently, a second fully automated M22-based TRAb immunoassay has been developed and is now commercially available (RAD 120; Radim, Pomezia, Italy). Here we report the results of a collaborative study performed in Italy for the evaluation of the analytical and clinical performance of this new analytical system in routine clinical laboratories, in comparison with other receptor-based immunoassay methods.

Materials and methods

The RAD 120 anti-TSHR fluorescent immunoassay uses a preformed immunocomplex based on native porcine TSHR solubilized from a thyroid cell membrane preparation and anti-porcine TSHR mouse mAb coated on magnetic microparticles. The capture antibody binds to the C-terminal moiety of the porcine TSHR which does not interfere with the binding of TRAb or M22 mAb to the TSHR. After addition of coated microparticles and alkaline phosphatase-labelled M22, serum TRAb bound to the TSH receptor are detected by their ability to inhibit the binding of labelled M22. The signal of the labelled M22 is expressed as the intensity of fluorescence generated by the substrate 4-methylumbelliferone at 450 nm.

The RAD 120 anti-TSHR immunoassay was compared with a second-generation TRAb immunoassay (Lumitest TRAK human; Brahms, Berlin, Germany), which uses human recombinant TSHR coated on poly-

Table 1 Sensitivity and specificity for GD of three generations of TRAb assays

Assay	Sensitivity (%)		Specificity (%)		References
	Mean	Range	Mean	Range	
First generation	77.5	52.2–94.0	99.2	97.5–100.0	10, 12–20
Second generation	95.9	72.6–100.0	97.9	91.4–100.0	15, 17–19, 22–30
Manual third generation	94.5	85.5–99.6	98.4	95.8–100.0	24, 26, 28–31
Automated third generation	97.7	96.0–100.0	99.5	98.9–100.0	25, 26, 33–35

styrene tubes. This assay is based on the ability of TRAb to inhibit TSHR binding by labelled bovine TSH.

Additionally the RAD 120 anti-TSHR immunoassay was also compared with a manual third-generation M22 antibody-based TRAb enzyme-immunoassay (anti-TSHR; Radim, Pomezia, Italy). This assay uses porcine TSHR immobilized in microplate wells. All assays were performed according to the manufacturers' instructions.

Patient samples were collected at the three participating centres (Latisana, Pordenone, Genova) following ethical guidelines. Altogether, 594 individuals were included in the study. Of these 594, 82 were suffering from GD (77% women; mean age 45.6 years, range 7–83 years), 57 from autoimmune thyroiditis (AIT, 91% women; 44.5 years, 14–78 years) and 292 from HT (80.5% women; 47.6 years, 14–82 years). The remaining 163, 106 healthy subjects (HS, 76.7% women; 46.5 years, 13–84 years) and 57 patients with infectious diseases (ID, 79% women; 39.6 years, 11–69 years) without any history of thyroid disease, served as control subjects.

The criteria for GD were based on initially documented HT with or without ophthalmopathy and ultrasound hypoechogenicity and increased thyroid blood flow. The criteria for HT were TSH <0.3 mIU/l, whereas the criteria for AIT were TSH >4.0 mIU/l and the presence of positive anti-thyroperoxidase antibodies. The criteria for ID were clinical symptoms and the presence of specific IgG and IgM antibodies against *Toxoplasma gondii*, rubella virus, Epstein-Barr virus, syphilis, hepatitis B and C virus, and cytomegalovirus.

Serum concentrations of TSH (reference range 0.3–3.0 mIU/l, low detection limit 0.003 mIU/l), free T_4 (range 0.88–2.20 ng/ml) and free T_3 (range 1.2–4.5 pmol/l) were measured using commercially available chemiluminescence assays (Centaur; Siemens HealthCare Diagnostics, Frimley, Camberley, UK). To obtain the positivity threshold, ROC analysis was performed [36]. Sensitivity (true-positive results) was calculated from GD patients, whereas specificity (true negative results) was calculated from healthy controls and ID patients.

The results were compared using Student's *t*-test calculated with Prism software (GraphPad Software, San Diego, CA). Correlation analysis was performed with Spearman's test. A P value <0.05 was considered statistically significant. Regression analysis was done according to the method of Passing and Bablok.

Results

Intraassay imprecision (coefficient of variation, CV) of the RAD 120 anti-TSHR immunoassay were between 3.4% and 10.0%. Interassay CV ranged from 11.9% to 14.6%. The functional sensitivity of the assay, defined as the lowest concentration of TRAb that could be measured with an intraassay CV of 20%, was determined to be 0.40 IU/l (Fig. 1).

In order to calculate the sensitivity and specificity of the assay, we first performed the ROC plot analysis, reevaluating the manufacturer's cut-off value for TRAb positivity. Altogether, 163 healthy subjects and ID patients were included for calculating specificity and 82 patients with untreated GD were included for calculating sensitivity. The area under the curve (AUC) for the automated RAD 120 TRAb assay was 0.994 (confidence interval 0.985–1.000), and the cross-point of highest specificity and sensitivity was 1.25 IU/l (Fig. 2). These data are in accordance with the manufacturer's proposed threshold for TRAb positivity at 1.5 IU/l, with a 'grey' zone between 1.0 and 1.5 IU/l.

With the goal of estimating the validity of the new automated TRAb assay, we compared this method with a

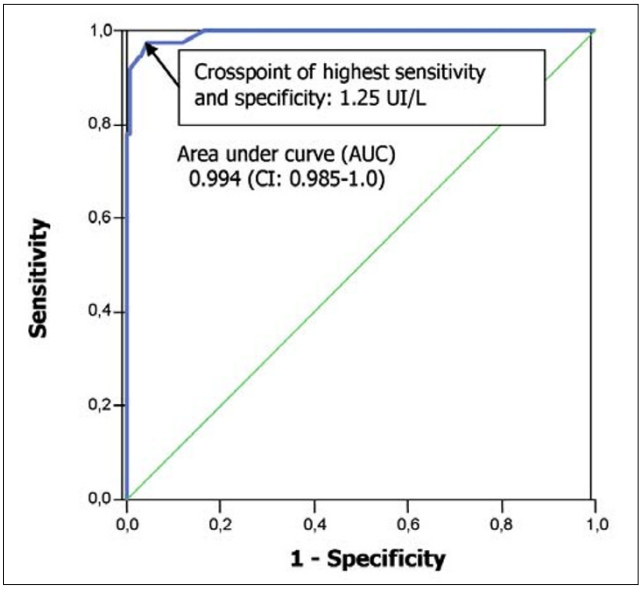

Fig. 1 ROC plot including GD patients, ID patients and healthy subjects

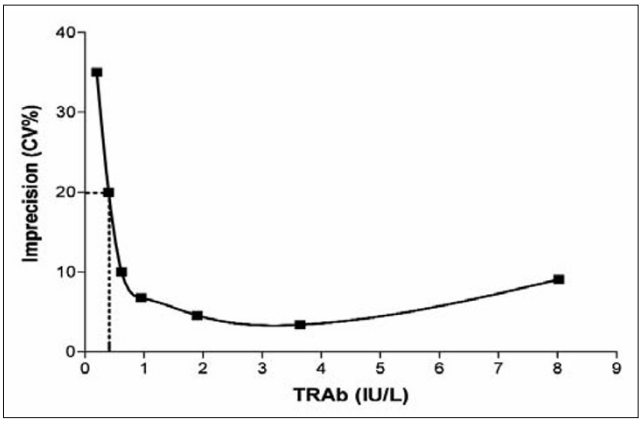

Fig. 2 Imprecision profile of TRAb automated RAD 120 immunoassay

well-established TRAb second-generation assay method (Lumitest, Brahms) and with a manual third-generation immunoassay produced by the same manufacturer as the automated method (Anti-TSHR, Radim). The positivity thresholds of these two methods were different from that of the automated method. The Lumitest method showed an optimal threshold value of 1.99 IU/l [23], while the value proposed by the manufacturer (Radim) for the manual third-generation method is 0.4 IU/l. Since comparison data were available only from the GD group, only the 82 GD patients were included in this analysis. Both assay systems showed a strong correlation, with *r* values of 0.86 (Lumitest) and 0.91 (Anti-TSHR).

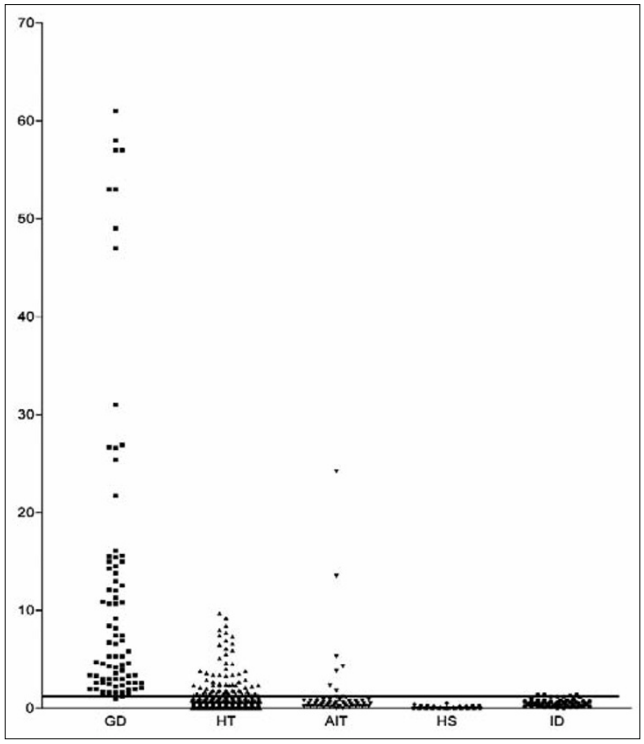

Fig. 3 Distribution of TRAb values obtained in all patients using the RAD 120 M22-based automated immunoassay (*GD* Graves' disease, *HT* hyperthyroidism, *AIT* autoimmune thyroiditis, *HS* healthy subjects, *ID* Infectious diseases)

In order to evaluate the automated assay under clinical conditions, we also measured TRAb levels in patients with different diseases or symptoms including GD, HT and AIT. The distributions of TRAb values obtained in all individuals in the present study are shown in Fig. 3. There was a clear difference in the distribution between healthy controls and GD patients. Using the 1.25 IU/l threshold value, the sensitivity was 97.6% and specificity 96.7%, respectively. Only two GD patients had values of TRAb below the cut-off value, and only five ID patients showed values above the threshold. Considering only the healthy subjects, the specificity was 100%. Using the cut-off values suggested by the respective manufacturers, the sensitivity of the two compared methods was 95.1% (Lumitest) and 96.3% (Anti-TSHR) (Table 2).

In addition, we also investigated the distribution of results in patients with AIT and in patients with HT. With the new automated assay, 6 of 57 patients (10.5%) in the first group and 68 of 292 (23.2%) in the second group were positive for TRAb. With the second-generation TRAb assay, only 4/57 patients (7.0%) and 55/292 patients (18.9%) were positive (Table 3).

Discussion

TRAb detection is widely accepted as a routine test for diagnosing and monitoring GD and for differential diagnosis of the various forms of HT. The aim of the present study was to evaluate the analytical sensitivity and precision and the diagnostic accuracy of a new fully automated system for the measurement of TRAb. Our results clearly show

Table 2 TRAb positivity in all individuals as determined with the three methods for TRAb detection

Assay	GD (*n*=82)	HT (*n*=292)	AIT (*n*=57)	HS (*n*=106)	ID (*n*=57)
RAD 120 positive	80 (97.5%)	68 (23.2%)	6 (10.5%)	0 (0%)	5 (8.8%)
Manual second generation	78 (95.1%)	55 (18.9%)	4 (7.0%)	–	–
Manual third generation	79 (96.3%)	–	–	–	–

Table 3 Analytical sensitivity and positivity threshold of three generations of TRAb assays

Assay	Sensitivity (IU/l)		Cut-off (IU/l)		References
	Mean	Range	Mean	Range	
First generation	3.2	2.4–4.0	nd	nd	10, 12–20
Second generation	1.4	0.8–3.0	1.4	1.0–4.0	15, 17–19, 22–30
Manual third generation	0.8	0.3–1.8	1.72	1.50–1.86	24, 26, 28–31
Automated third generation	0.8	0.7–0.9	0.7	0.4–1.0	25, 26, 33–35

nd not determined

that the analytical (functional) sensitivity of the RAD 120 system (0.4 IU/l) was higher than the mean of the second-generation (1.4 IU/l) [15, 19, 22, 23], manual third-generation (1.1 IU/l) [26, 30] and other automated third-generation assays (0.8 IU/l) [32, 37], probably because of the high analytical precision of the new system (Table 3).

Within the last two decades huge efforts have been made to improve the first-generation methods for the measurement of TRAb, based on competitive binding of patients' TRAb and labelled bovine TSH to a soluble porcine TSHR [2]. After cloning of human TSHR, a second-generation TRAb assay with high diagnostic accuracy was launched onto the market. Most recently, a completely new method for manual measurement of TRAb (third generation) has been described, which is based on the binding of a labelled human mAb M22 to porcine TSHR. The drawback of all these manual assay systems is their labour-intensive and time-consuming procedures. The great advantage of the fully automated TRAb detection systems is the reduction in the number manual procedures, coupled with the possibility of integration of the assay into the workflow on routine laboratory analysers without splitting patients' samples.

We also re-evaluated the positivity threshold level for TRAb RAD 120 as recommended by the manufacturer. Based on the ROC analysis a cut-off value for TRAb positivity of 1.25 IU/l had the highest sensitivity and specificity for diagnosing GD. This value is in the middle of the 'grey zone' proposed by the manufacturer (1.0–1.5 IU/l). The results are similar and strictly comparable to the cut-off values obtained in recent studies with other manual or automated third-generation routine assays (0.4–1.86 IU/ml) (Table 3).

In our experience, despite calibration against the international reference preparation IRP 90/672, TRAb values obtained with the comparison methods (Brahms and Radim) were 1.5 times higher and 1.5 lower than those of the RAD 120, respectively (data not shown). The reasons of these discrepancies are not clear. When corrected for the cut-off values, the results of the three methods are similar, with some individual differences. This confirms a recent report stating that different TRAb assays employing different ligands and ligand partners may have similar sensitivities and specificities but are not interchangeable [29].

The new automated TRAb assay showed diagnostic sensitivity comparable to other automated third-generation TRAb assays (97.6% vs 97.7%), but higher than the manual third-generation (94.5%) and classical second-generation TRAb assays (95.9%). The specificity was high and comparable to those of the other third and second-generation methods (100%, 98.5%, 98.9%, respectively). Also in our experience the RAD 120 assay showed diagnostic sensitivity higher than those of manual third-generation and manual second-generation assays (Table 1).

Interestingly, we found that 6 of 57 AIT patients (10.5%) showed low/intermediate concentrations of TRAb, confirming early and recent studies in which 5–15% of AIT patients were TRAb-positive [15, 17, 19, 24, 27, 30, 31, 33]. This finding is probably because of the presence of TSH blocking or TSH-binding neutral antibodies that inhibit the binding of M22 mAb [38].

We also found that 23.2% of patients with HT were TRAb-positive. This figure is higher than that observed with the second-generation assay (Table 3). Thus, the new assay appears to be more sensitive than the standard TRAb method in hyperthyroid patients with either GD or multinodular toxic goitre, or even in L-thyroxine-treated patients. Indeed, TRAb positivity in patients with HT associated with disorders other than GD has been described in some reports [18, 30], particularly in patients with multinodular toxic goitre (in which GD develops on top of a euthyroid multinodular goitre in areas of low iodine intake), and in patients with autoimmune thyroiditis treated with high-dose L-thyroxine therapy (Table 4).

At the time of this report, eight patients with TRAb RAD 120 positivity were still undiagnosed, and probably a little uncertainty concerning misclassification remained particularly in patients with borderline or low positive TRAb concentrations.

In summary, our results show that this new automated assay system for the measurement of TSH receptor autoantibodies has a high sensitivity for detecting GD and a high specificity for differentiating this autoimmune disease from other hyperthyroid disorders. Moreover, this assay is less time-consuming and labour-intensive and is therefore the up-to-date technology for TRAb detection in clinical practice.

Conflict of interest statement The authors declare that they have no conflict of interest related to the publication of this article.

Table 4 TRAb-positive hyperthyroid patients

Patients	RAD 120	Lumitest
Graves' disease	33	30
Multinodular goitre	23	20
Autoimmune thyroiditis with high-dose L-thyroxine therapy	4	2
Undiagnosed	8	7
Total	68	59

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