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Immunoassay of thyroid peroxidase autoantibodies: diagnostic performance in automated third generation methods. A multicentre evaluation

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Abstract

Background: The use of automated immunometric methods for the detection of anti-thyroid peroxidase antibodies (TPOAb), the main serological marker of autoimmune thyroid diseases (AITD), has expanded in recent years. However, it is not known whether these new automated platforms have improved the diagnostic performance of TPOAb assays. The aim of this study was to evaluate the potential improvement of the inter-method agreement of current automated third generation systems, 12 years after a previous study, which had assessed the analytical variability between semi-automated second generation methods of TPOAb detection.
Methods: Eight pools of sera from patients with chronic lymphocytic thyroiditis, exhibiting different TPOAb concentrations, were collected from routine laboratory diagnostics and distributed to seven companies throughout Italy. All automated third generation methods were calibrated against the Medical Research Council (MRC) reference preparation 66/387.
Results: The overall mean variability (CV) was 93.6% when results were expressed in part as arbitrary Units (U/mL) and in part as International Units (IU/mL). The conversion of all values in IU/mL resulted in a significant decrease of CV (49.8%). The CV expressed as COM (cut-off concentration multiples) was 64.0%. Agreement of qualitative results was 95.3% with a pronounced difference in the threshold values proposed by manufacturers (range 3.2–35.0 IU/mL).
Conclusions: These findings confirm the improvement of harmonisation between different methods of automated third generation TPOAb assays. Nevertheless, further efforts should be made in the definition of the positive cut-off concentration to avoid misclassification of AITD patients as well as in a new international reference preparation and in the autoantigen purification modality.

Keywords: anti-thyroid peroxidase antibodies; autoimmune thyroid disease; automated platforms; harmonisation; immunoassay.

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Introduction

Autoimmune thyroid diseases (AITD) are the most frequent organ-specific autoimmune diseases in the world, affecting 5%–10% of the population. The clinical picture of AITD consists of chronic lymphocytic thyroiditis [i.e., Hashimoto's thyroiditis (HT)] and primary hyperthyroidism [i.e., Graves' disease (GD)], in which the entire spectrum of thyroid function disorders is represented [1].

The diagnosis of AITD is based on the measurement of the circulating thyroid specific autoantibodies. This is undertaken in addition to clinical representation and functional/imaging data, as is common practice for other autoimmune diseases. Amongst the known types of thyroid autoantibodies, anti-thyroid peroxidase (TPOAb) and anti-thyrotropin receptor (TRAb) antibodies are hallmarks for the diagnosis of AITD [2, 3], as confirmed by recent guidelines for the clinical management of thyroid diseases [4–8].

TPOAb are the main serological marker of AITD, as they are detected in the sera of the majority of patients with GD (>80%), HT (>90%) and post-partum thyroiditis (>70%). However, they are also detectable in 10%–20% of healthy subjects [2, 9]. In AITD patients' sera TPOAb are predominantly IgG of all subclasses, with high levels of concentration (up to mg/mL) [10].

In recent years, the methods for the measurement of circulating autoantibodies and in particular of TPOAb have markedly evolved encompassing three generations of analytical technologies [11]. The use of sensitive automated third generation immunometric methods has progressively expanded in clinical laboratories [1, 12–14]. However, it is not known to what extent these new platforms have improved in terms of diagnostic accuracy with respect to the former methods. Due to the known problems of variability and low level of standardisation, several authors have underlined the need to assess the analytical performance of commercially available TPOAb assays [7, 12].

Twelve years ago, the Study Group on Autoimmunology of the Italian Society of Laboratory Medicine (SIMeL) demonstrated a high analytical variability between methods for the detection of TPOAb in a collaborative study with biomedical industries [15]: at that time, 10 different semi-automated second generation methods were assessed. In order to evaluate potential improvement of the inter-methods agreement of current automated third generation systems, the study has been replicated with eight fully automated systems for TPOAb detection produced by seven participating companies.

Materials and methods

Eight pools of sera from patients with chronic lymphocytic thyroiditis, exhibiting different TPOAb concentrations, were collected within 1 month from routine laboratory diagnostics, subdivided into aliquots of 0.5 mL and stored at –80 °C.

Patients were diagnosed according to typical thyroid ultrasound pattern and thyroid-stimulating hormone (TSH) increase, above the upper reference limit of 4.0 mU/L. Enrolled subjects gave informed consent for participation in the study.

The aliquots were distributed to seven companies that produce analytical systems and reagents for TPOAb assay: Abbott Laboratories (Chicago, IL, USA), Beckman-Coulter (Brea, CA, USA), DiaSorin (Saluggia, Italy), Roche Diagnostics (Mannheim, Germany), Siemens Healthcare Diagnostics (Erlangen, Germany), Thermo Fisher Scientific B.R.A.H.M.S (Hennigsdorf, Germany), Tosoh Corporation (Tokyo, Japan). In total, eight third generation methods/kits (5 CLIA, 1 ECLIA, 1 TRACE, 1 FEIA) were employed (Table 1). Each participating company was invited to conduct the determination in two of their own laboratories and/or public hospital laboratories for a total of 16 laboratories throughout Italy (see Supplemental Data, Table 1). All the immunometric methods were calibrated against the reference Medical Research Council (MRC) preparation 66/387 and implemented in automated instruments. Native (3/8) or recombinant (5/8) antigens coated on the solid-phase were used for TPOAb assay.

Quantitative results were expressed in International Units (IU/mL) and in multiples of the threshold value (cut-off multiples: COM), calculated as the ratio between each analytical result and the proposed cut-off concentration (both expressed in IU/mL). In two of the eight methods/kits, results were initially expressed in arbitrary Units (U/mL) as suggested by the manufacturers and subsequently in IU/mL, obtained by introducing conversion factors. Concordance between results was calculated from the qualitative data (positive/negative).

Data were reported as mean and range (min-max) for each method. The variability between the two laboratories using the same method (intra-method variability) and the total variability between methods (overall variability) were expressed in terms of coefficient of variation (CV). Differences between absolute values and their corresponding COMs were determined by paired Student's t-test. p-Value <0.05 was considered statistically significant for all tests (GraphPad Prism Software, version 4.0, San Diego, CA, USA).

Results

The overall variability of the quantitative results was 93.6% (range 62.0%–118.1%) (Figure 1) (see Supplemental Data, Table 2). Two methods/labs (E and G) clearly

Table 1 Participating companies, instruments and methods used for TPOAb detection.

Company	Instrument	Method/Tracer	Code
Abbott, USA	Architect (i1000-i2000)	CLIA/Acridinium esters/salts	**A**
Beckman Coulter, USA	Unicel (Dxl 800-DxC 880i)	CLIA/Dioxetane phosphate	**B**
DiaSorin, Italy	Liaison	CLIA/Isoluminol derivatives	**C**
Roche Diagnostics, Germany	Cobas 8000-Modular E	ECLIA/Ruthenium bis-pyridyl	**D**
Siemens HD, Germany	Centaur XP	CLIA/Acridinium esters	**E**
Siemens HD, Germany	Immulite 2000 XPi	CLIA/Dioxetane phosphate	**F**
Thermo Fisher BRAHMS, Germany	Kryptor-Kryptor Compact	TRACE/Europium cryptate	**G**
Tosoh Corporation, Japan	AIA 2000	FEIA/4-methylumbelliferyl phosphate	**H**

CLIA, chemioluminescent immunoassay; ECLIA, electrochemiluminescent immunoassay; TRACE, time resolved amplified cryptate emission; FEIA, fluorimetric enzyme immunoassay.

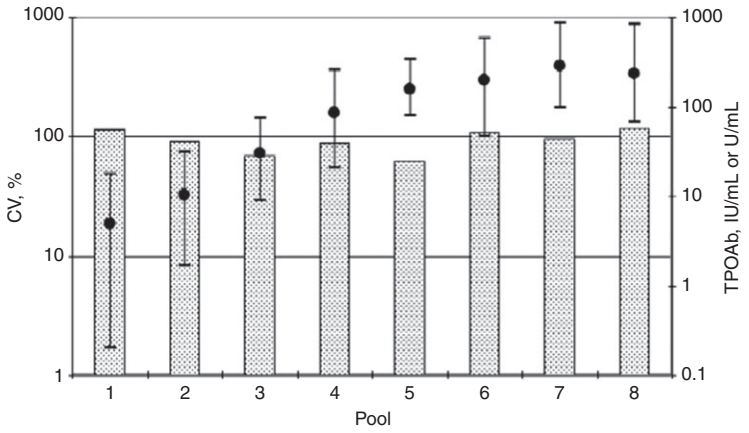

Figure 1 TPOAb results, expressed in IU/mL or U/mL in the eight pools before correction.
Columns represent the coefficient of variation and black circles the mean value with range. The overall mean CV of the quantitative results was 93.6% with a range of 62.0%–118.1%.

showed higher results than those of the other methods: a detailed analysis of the assay characteristics highlighted that the two methods/labs expressed the results in arbitrary Units (U/mL) instead of International Units (IU/mL). The correction of values in IU/mL, obtained by introducing a conversion factor (0.333 for the E method and 0.175 for the G method), resulted in a significant reduction of variability, that was 49.8% (range 23.1%–104.5%) (Figure 2), (see Supplemental Data, Table 3). Figure 3 illustrates the same results expressed as COM: the overall mean CV was 64.0% (range 36.2%–87.3%) (see Supplemental Data, Table 4). The difference between the two means was not significant (p=0.4) (Table 2).

The percentage agreement between qualitative results, subdivided according to sera and methods, is shown in Figure 4: overall concordance was 95.3% with three false negative results in pool 3 (see Supplemental Data, Table 5). Notably, there is a pronounced difference in the threshold values of positivity proposed by manufacturers, ranging from 3.2 IU/mL (H method) to 35.0 IU/mL (F method) (see Supplemental Data, Table 5).

The intra-method variability between each pair of laboratories is shown in Figure 5 and ranges from 2.6% (A method) to 15.7% (G method). Of note, the variability between methods tends to decrease with increasing TPOAb concentration (Figure 6).

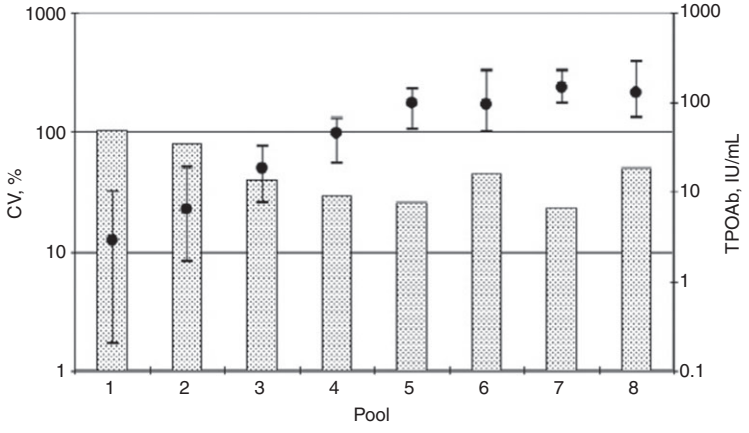

Figure 2 TPOAb results, expressed in IU/mL in the eight pools after correction.
Columns represent the coefficient of variation and black circles represent mean value with range. The overall mean CV of the quantitative results was 49.8% with a range of 23.1%–104.5%.

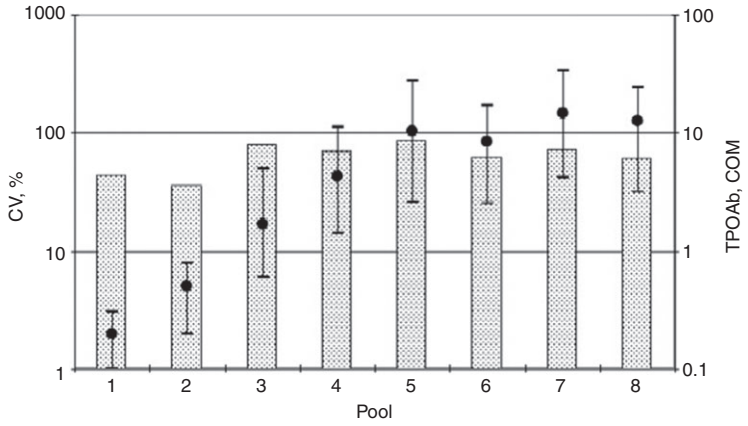

Figure 3 TPOAb results, expressed as cut-off concentration multiples (COM) in the eight pools.
Columns represent the coefficient of variation and black circles the mean value with range. The overall mean CV of the quantitative results was 64.0% with a range of 36.2%–87.3%.

Table 2 Comparison of overall mean variability calculated from results expressed in IU/mL and in COM.

Antibody	Mean CV, %	Min-Max, %	p-Value
TPOAb, IU/mL	49.8	23.1–104.5	n.s.
TPOAb, COM	64.0	36.2–87.3	

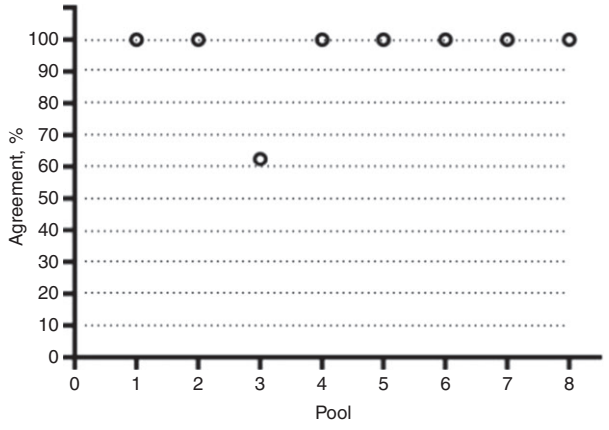

Figure 4 Agreement between qualitative results of TPOAb testing in the eight pools.
Overall concordance was 95.3%.

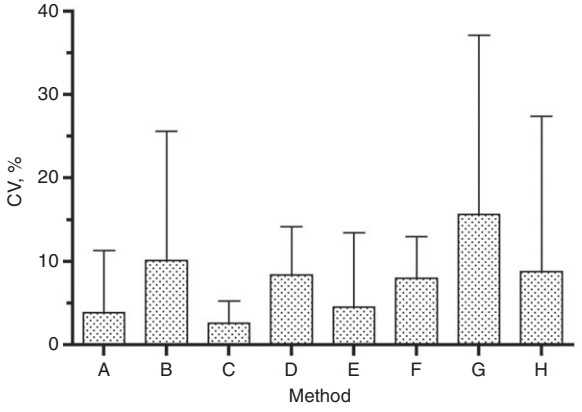

Figure 5 Intra-method variability between laboratories.
Intra-method variability (CV) ranged from 2.6% to 15.7%. Data were expressed as mean and range (min-max).

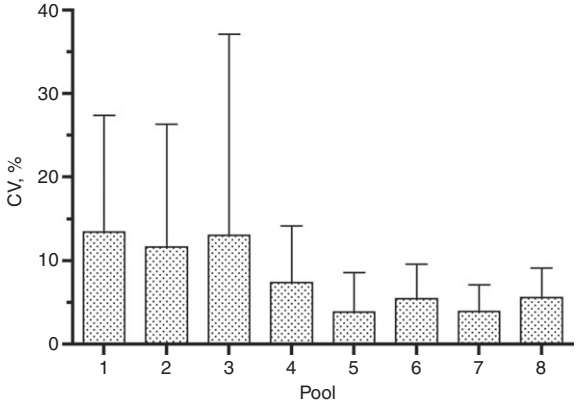

Figure 6 Variability between methods in relation to TPOAb concentration.
Variability (CV) between methods tended to decrease with increasing TPOAb concentration. Data were expressed as mean and range (min-max).

Discussion

About a decade ago, in the previous study, it was observed that "despite the improvement of current analytical procedures for TPOAb immunoassay derived by technology advancement, there was an unexpected and pronounced analytical variability in the quantitative results among

the various methods" [15]. In the present study, taking into account the technological evolution from second generation semi-automated to the recent third generation fully-automated platforms/systems for the TPOAb measurement, a significant improvement of the diagnostic accuracy and the harmonisation of the results was to be expected. The present results show, however, that the level of standardisation is still quite low. This is likely due to four main factors: a) the analytical variables of the assays; b) the use of international reference preparations; c) the definition of the positive cut-off concentration; and finally d) the autoantigen purification procedures.

In the present study, considering the results calculated according to manufacturers' indication, the overall variability of the automated method for TPOAb measurement is even higher than in the first study: 93.6% vs. 76.0% [15]. It is worth noting that the laboratories of two manufacturers use arbitrary units derived from secondary 'in house' standards instead of the International Units of the reference MRC preparation 66/387. This source of variability (b factor, see above) significantly affects the agreement of results between methods. In fact, by introducing appropriate correction factors, the analytical variability decreases from 93.6% to 49.8%. The combined amelioration of b factor and a factor, which largely depends upon the degree of automation [15], results in an improved harmonisation.

Regarding the c point (the definition of cut-off, as the upper reference limit) there is no doubt that this factor strongly affects the variability between methods. As a matter of fact, the variability increased from 62.9% in the earlier study to 67.0% of the present study. The cut-off values proposed by manufacturers do not always fulfil the international recommendations, which suggest the use of direct methods on a reference sample of 120 young male adults [7], nor are produced by means of indirect methods on current data from routine diagnostics stored in the laboratory databases, as proposed [16]. These approaches could be adopted by every clinical laboratory, as a two-step strategy [16]: this might allow a harmonisation of the different proposed cut-offs, currently ranging from 3.2 to 35.0 IU/mL with a 10-fold variation between minimum and maximum, as indicated also in previous studies [15–17]. Consequently, the concordance of qualitative results, slightly lower with respect of the earlier study (95.3% vs. 96.6%), could increase by reducing misclassification of patients with AITD.

Taking into account that the contribution of the intra-method variability (from 2.6% to 15.7%) (see Supplemental Data, Table 5) to overall variability is reduced with respect to the previous study, the residual variability of 30% between the different automated TPOAb assays could lie in the autoantigen purification and in the different representation of immunodominant epitopes of TPO on the solid phase. It is now demonstrated that polyclonal TPOAb present in the sera of patients with AITD are heterogeneous and react with several B-cell conformational and linear epitopes on the surface of TPO.

TPO is a large membrane-associated glycoprotein, composed of a propeptide, a myeloperoxidase (MPO)-like domain (142-738 amino acid residues), a complement control protein (CCP)-like domain (739-795 amino acid residues), an epidermal growth factor (EGF)-like domain (796-841 amino acid residues), a single transmembrane region and a short intracellular tail [2, 18]. The MPO, CCP and EGF-like domains share a high sequence homology with MPO, CCP and EGF proteins, respectively. Two different hinge regions are located between the MPO- and CCP-like domains and CCP- and EGF-like domains [2].

Two overlapping domains, termed A (IDR-A) and B (IDR-B), constitute the immunodominant region (IDR) of TPO [19]. However, the immunological response to other regions of TPO (non-A/non-B IDR) may occur in patients with AITD or non-autoimmune thyroid diseases: these epitopes are linear and formed by continuous amino acid sequences [2, 9, 10, 20–24]. The distribution of IDR-A-, IDR-B, and non-IDR-A/IDR-B antibodies is approximately 25%, 50% and 25%, respectively [2, 20]. These regions are dependent upon the densely folded structure of TPO, with the MPO- and CCP-like domains lying in close proximity: the IDR forms a single complex on TPO, centred around residues 599-617 within the MPO-like domain, whereas the EGF-like domain, transmembrane region and propeptide may not be involved in antibody binding. However, the CCP- and EGF-like domains and their 'hinge' region help maintain the three-dimensional structure of TPO required for antibody binding [2, 9, 22, 24].

TPO can be prepared in small amounts from human thyroid tissue, particularly hyperplastic thyroids. Using recombinant technology, TPO can be obtained in larger amounts from eukaryotic cells, such as Chinese hamster ovary cells. A eukaryotic cell is required because autoantibodies predominantly recognise conformational epitopes. As TPO is a membrane-associated protein, its purification requires detergent solubilisation and limited tryptic digestion of the thyroid microsomal fraction. Further purification can be obtained by affinity purification using monoclonal antibodies to human TPO. Commercial kits generally contain purified TPO prepared by processes which are trade secrets [25].

It is conceivable that the different coating preparations of autoantigen (natural or recombinant) on solid

phases affect the proper exposure of the immunodominant epitopes recognised by the polyclonal antibodies present in serum of AITD patients. This may result in a lack of recognition/identification of some of them by the different methods [26, 27].

The results of this study confirm in part the expected improvement of harmonisation between the different methods of automated third generation TPOAb assay, which over the past 10 years have become popular in clinical laboratories, replacing the semi-automated methods of the second generation. Further efforts must be made in the definition of the threshold values of positivity to avoid misclassification of patients with AITD.

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