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# In-House Solid-Phase Radioassay for the Detection of Anti-thyroglobulin Autoantibodies in Patients with Differentiated Thyroid Cancer

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**Abstract** Thyroglobulin autoantibodies (TgAb) are estimated to detect potential interferences in thyroglobulin (Tg) immunoassays and also for the diagnosis of autoimmune thyroid disease. A user friendly and robust in-house solid-phase radioassay was standardized and parameters like sensitivity, reproducibility and stability were assessed. Further, it was validated and evaluated for the detection of autoantibodies in differentiated thyroid cancer (DTC) patients. Totally 301 samples received in our laboratory for routine serum Tg estimation were studied. The samples were analyzed for TgAb by the solid-phase radioassay developed in-house and compared with commercial anti-hTg IRMA kit (Immunotech, France). The control group comprised of 37 euthyroid males from our Centre. The intra- and inter-assay CVs for the two quality control samples (Control A = 104 ± 12.6 IU/mL and Control B = 1029 ± 114 IU/mL) were found less than or equal to 6.05 and 13.85 % respectively. Solid-phase radioassay showed a good agreement on comparison with Immunotech IRMA (r = 0.99). Using the proposed cut-off thresholds (in-house solid-phase radioassay 52 IU/mL and Immunotech IRMA 30 IU/mL), 5.4 % of the control subjects were positive for TgAb by both the methods. Prevalence of TgAb in DTC patients was 17.3 and 16.6 % using the Immunotech kit and in-house solid-phase radioassay respectively. The in-house solid-phase radioassay has the requisite sensitivity for the evaluation of TgAb comparable to commercial kit and also suitable for routine use as it is rapid, user friendly and economical.

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## Introduction

Circulating anti-thyroglobulin autoantibodies (TgAb) and anti-thyroid peroxidase (TPO-Ab) are the early indicators of autoimmune thyroid diseases (AITD) [1–3]. Further, serial serum TgAb measurement may be an independent prognostic indicator of the efficacy of treatment for, or recurrence of, DTC in TgAb positive patients [4–6]. The reliable detection of TgAb is critical because the prevalence of TgAb in DTC patients is high (10–25 %) [1, 4–6]. Circulating TgAb is known to interfere with serum Tg measurements in a method-dependent manner [7]. Therefore, TgAb measurement in all samples is recommended prior to Tg analysis because even low TgAb titers may have unpredictable effects on Tg results [8]. The National Academy of Clinical Biochemistry (NACB) guidelines recommends the use of serial TgAb measurement in complement with serum Tg estimation every 6–12 months after surgery [9]. NACB has also suggested a criteria for establishing the reference limits for thyroid autoantibodies in male subjects those are young, biochemically euthyroid, with no goiter and no family history of AITD, because females are more likely to have undiagnosed thyroid disease [10].

There has been a striking improvement during few years in the techniques used for the measurement of TgAb. Earlier insensitive immunofluorescence or hemagglutination methods have been replaced by more sensitive immunoassays like RIA and ELISAs [11]. Despite the marked improvements in the analytical techniques, inter-method variability still exists. Also in spite of the use of

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WHO International Reference preparation (IRP 65/93) for assay standardization, the inter-method variability has not decreased as expected [10].

Our Centre is one with major facilities for treating thyroid cancer patients with radioactive $^{131}$I and follow-up of these patients is regularly performed by serum Tg estimation. As TgAb are known to interfere in the measurement of serum Tg, in the present study, an in-house solid-phase radioassay for serum TgAb measurement was developed. The assay was validated and the results obtained were compared with a commercial anti-hTg IRMA kit.

## Materials and Methods

### Study Subjects

To establish normal reference intervals, serum samples from 37 apparently healthy male subjects (staff members and students of our Centre, who had no known history of thyroid disorders) were used. No one received any medication with a known influence on the thyroid function other than the normal use of iodized salt. The study subjects were a total of 301 patients (females:201, males:100) of DTC referred to the Radiation Medicine Centre post-operatively (after thyroid surgery). Serum specimens were collected from these patients for routine Tg analysis and TgAb levels measured. Patient consent was taken.

### Human Thyroglobulin

Tg was extracted from thyroid tissue of human origin within 4–6 h of autopsy and purified according to the method of Mouriz and Stanbury [12].

### *Immobilization of Tg on Polystyrene Solid-Phase*

Clean and dry polystyrene tubes (70 mm × 10 mm) were chemically activated overnight using 500 μL of 1.5 % glutaraldehyde solution (v/v) in 0.1 M NaHCO$_3$ buffer, pH 8.6, at room temperature. Subsequently, the glutaraldehyde solution was aspirated out and all the tubes were washed twice with 2 mL of 0.1 M NaHCO$_3$ buffer. These tubes were coated with 25 μg of purified hTg (in 0.5 mL of 0.1 M borate buffer, pH 8.0) and incubated overnight. The solution was aspirated to remove unbound Tg and the tubes were washed twice with 2 mL of 0.1 M NaHCO$_3$ buffer. The empty sites on the polystyrene surface were then blocked with 0.5 mL of 0.1 M Tris-buffer, pH 8.2 containing 0.2 % ethanolamine for 2 h. The solution was aspirated, and the tubes were again washed with 2 mL of 0.1 M Tris buffer. The tubes were further blocked with 0.5 mL of 0.1 M Tris containing 0.2 % BSA (w/v) buffer for an overnight period. The blocking solution was aspirated and the tubes were air dried for 3–4 h and stored in airtight zip sealed plastic bags, at 4 °C.

### *Radioiodination of Protein A ($^{125}$I-PA)*

Protein A from *S. aureus* (Cowan strain was procured from Sigma Chemical Company, St. Louis, USA) was labeled with $^{125}$I by the Iodogen method [13]. The specific activity of $^{125}$I-Protein A varied from 2.0 to 2.2 GBq/μg. The immunoreactivity of the tracer was verified by determining the percent non-specific binding (%NSB) with anti-Tg antibody at a concentration of 0 IU/mL and a maximum binding (%Bmax) at highest standard concentration of 3100 IU/mL for each batch of Protein A that was used for labeling. The shelf-life of the Tg-coated tubes was studied by assaying its binding ability (%Bmax), as a function of time over several weeks.

### *Solid-Phase Radioassay Procedure*

The solid-phase radioassay was optimized for various parameters including the incubation time and temperature, using the following procedure. The calibrators, controls and test samples (20 μL) were added in the Tg coated tubes and the final assay volume was made to 500 μL with the PBS-EDTA buffer (0.025 M PO$_4$; 0.15 M NaCl; 0.01 M EDTA; 0.01 % NaN$_3$; pH 7.5). The tubes were incubated for 3 h in a shaking water bath at 37 °C. After the incubation, the contents of the tubes were aspirated and washed twice with the wash buffer (0.025 M PO$_4$; 0.15 M NaCl; 0.01 % NaN$_3$; 0.05 % Tween 20, pH 7.5) and 500 μL of $^{125}$I-Protein A, with an activity of ∼3000 Bq was added to each tube and incubated for 90 min in a shaking water bath at 37 °C. After the incubation, the contents of the tubes were aspirated and the tubes washed as before so as to remove unbound tracer. The bound radioactivity was then determined in a gamma counter. The concentrations in the samples were obtained by interpolation from the standard curve obtained by plotting cpm for each standard versus the corresponding concentration of TgAb. The concentration of TgAb in the samples was directly proportional to the radioactivity.

For calculating the sensitivity of the assay, the dose–response curve was constructed on linear graph paper. The standard deviation in the measurement of zero antigen concentration (Bo) was calculated from a set of 10 values. The dose corresponding to a response three standard deviations away from the mean zero dose response (mean Bo + 3SD) was extrapolated from the graph and taken as the theoretical sensitivity with 99 % confidence. Replicates of two quality control sera were set-up in a single assay as

well as in assays carried out at different intervals to illustrate the intra- and inter-assay precision. The Tg coated tubes were stored at 4 °C, and the stability tested by the protocol described above for a total period of 15 months. Percent Bmax and %NSB were used as an index of stability of the immobilized Tg.

### Comparison of TgAb Levels Obtained by In-House and Commercial Assay

Random 301 patients with DTC were analyzed for the incidence of TgAb using the solid-phase radioassay described here and also by the commercial Anti-hTg IRMA kit (Immunotech, France). According to the NACB recommendations, both the assays are calibrated against the WHO 1st International Reference Preparation (IRP) 65/93. A linear regression analysis was performed for the data obtained by both the methods. Scatter diagram was drawn for the TgAb concentrations obtained by these methods and a correlation coefficient 'r' was also calculated. The Immunotech TgAb assay was performed according to the manufacturer's instructions. The upper reference limit provided by the manufacturer of commercial kit was at 30 IU/mL.

### Statistical Analysis

Pearson's coefficient of correlation and regression analysis were used for the data analysis. P < 0.05 was considered as statistically significant.

## Results

### Immunoreactivity and Shelf-Life of $^{125}$I-Protein A

The % Bmax of $^{125}$I-Protein A ranged from 35 to 40 % with a NSB of <1.0 %. A single $^{125}$I-Protein A preparation having a specific activity of 2.07 GBq/μg was checked for its binding ability i.e. % Bmax at intervals of 5, 13, 15, 22, 35, 43, 72 and 120 days post labeling. The percentage of $^{125}$I-Protein A bound was calculated and plotted against time (Fig. 1). $^{125}$I-Protein A did not show significant decrease in immunoreactivity for a period of 5 weeks. Thereafter, some loss in immunoreactivity was observed over a period of 4 months, but a satisfactory assay could still be obtained with this old tracer.

### Analytical Sensitivity and Assay Precision

For solid-phase radioassay the minimum detectable level at 3SD (99 % confidence) was 3.2 IU/mL and the intra- and inter-assay CVs were found less than or equal to 6.05 and
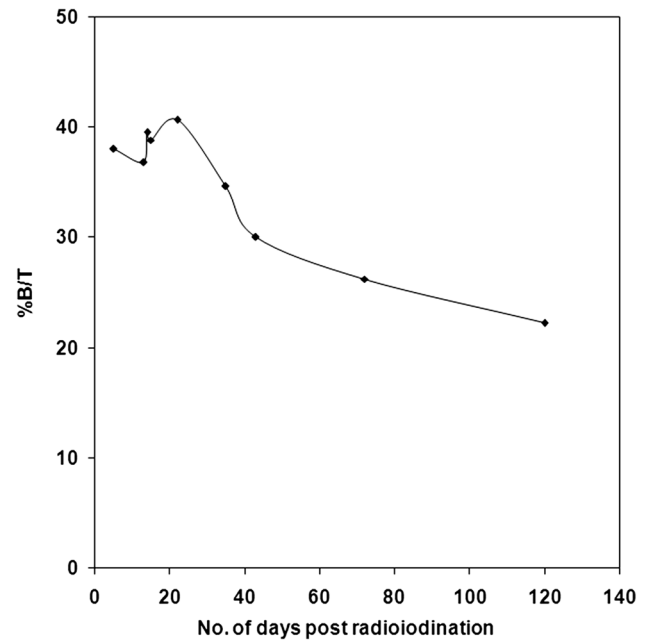


**Fig. 1** Shelf-life of $^{125}$I-Protein A post radioiodination

13.85 % respectively as summarized in Table 1. For Immunotech kit both intra- and inter-assay CVs were ≤15.0 % with sensitivity of 3.0 IU/mL.

### Assessment of Stability and Performance of the Tg Coated Tubes

The Tg-coated tubes when tested for their binding ability (%Bmax), did not show any significant loss in the binding capacity at least for a period of 1 month. Thereafter, up to a period of 15 months there was no significant fall in %Bmax (Table 2).

### Reference Ranges

The distributions of TgAb values in control sera are reported in Fig. 2a, b. Reference cut-offs were determined for each method using the estimated anti-Tg antibody concentration in samples from 37 male volunteers as per the NACB proposal [10]. The upper 95th percentile values for TgAb was 52 IU/mL and 30 IU/mL for the in-house solid-phase radioassay and the Immunotech anti-hTg IRMA kit respectively. With these reference ranges, two subjects (5.4 %) were scored positive for TgAb by both the methods (Fig. 4).

### Comparison Between Two Methods

The comparison of TgAb levels measured by the in-house solid-phase radioassay and the Immunotech kit is shown in Fig. 3. The correlation coefficient obtained was 0.99

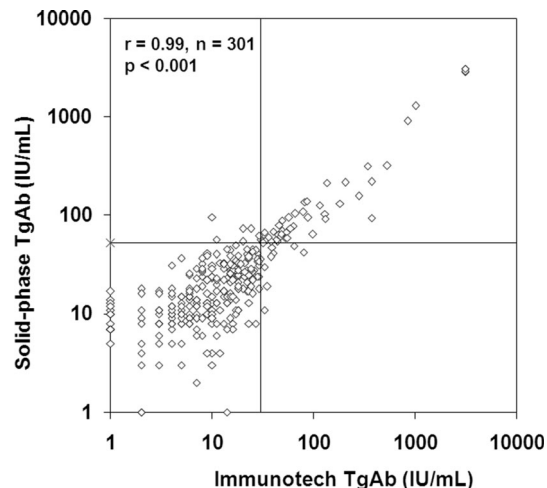**Table 1** Imprecision data of TgAb as measured by solid-phase radioassay

|  | With-in run | | | Between run | | |
|---|---|---|---|---|---|---|
|  | n | Mean ± 2SD (IU/mL) | CV (%) | n | Mean ± 2SD (IU/mL) | CV (%) |
| Control A | 10 | 104 ± 12.6 | 6.05 | 10 | 102 ± 28 | 13.85 |
| Control B | 10 | 1029 ± 114 | 5.5 | 10 | 1071 ± 224 | 10.5 |

**Table 2** Stability studies of Tg coated tubes with $^{125}$I-PA

| Parameter | Time lapsed after coating (days) | | | | | |
|---|---|---|---|---|---|---|
|  | 5 %B/T | 14 %B/T | 27 %B/T | 35 %B/T | 120 %B/T | 450 %B/T |
| NSB | 1.0 | 1.1 | 0.78 | 0.77 | 0.21 | 0.36 |
| Bmax | 36.8 | 40.6 | 34.6 | 24.2 | 22.2 | 19.5 |
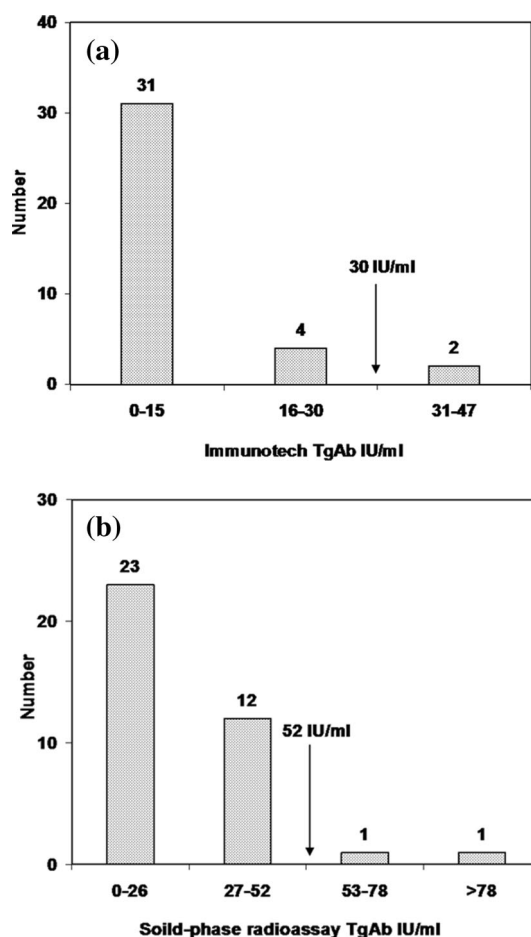


**Fig. 2** **a** TgAb results by Immunotech IRMA in 37 sera from control subjects. The *arrow* shows the cut-off value. **b** TgAb results by solid-phase radioassay in 37 sera from control subjects. The *arrow* shows the cut-off value



**Fig. 3** Scatter of TgAb levels obtained by solid-phase radioassay and Immunotech anti-hTg IRMA kit. The *vertical* and *horizontal lines* indicate the upper reference limits

concordance for TgAb methods was determined using our reference limits it was found to be 100 % for the control group (Table 3) and 94.68 % for DTC group (Table 4).

### TgAb in Patients with DTC

The TgAb prevalence by the two methods (alone or in combination) is shown in Fig. 4. Of the 301 patients, 52 samples (17.3 %) were positive for TgAb using Immunotech kit and 50 (16.6 %) by the solid-phase radioassay. The two methods yielded concordant positive TgAb results in 43 sera (14.29 %). Seven sera were found to be positive with the solid-phase method alone and 9 by Immunotech method alone.

### Discussion

Thyroglobulin (Tg), a glycoprotein synthesized in normal or malignant thyroid follicular cells, is an important marker for persistent tumor or recurrence in patients with differentiated thyroid cancer who have undergone total thyroidectomy and remnant ablation with radioiodine. However, in spite of Tg being the most sensitive marker, the presence of antithyroglobulin antibody (TgAb) interferes with measurement of Tg and leads to the

($p < 0.001$) and the linear regression equation observed was $y = 0.9681x + 7.699$, showed a strong method comparability between the two methods. When the diagnostic

**Table 3** Diagnostic concordance of TgAb in control group using the in-house reference intervals

| | Immunotech IRMA | | Total | Overall concordance |
|---|---|---|---|---|
| | NEG (≤30 IU/mL) | POS (>30 IU/mL) | | |
| Solid-phase radioassay | | | | |
| NEG (≤52 IU/mL) | 35 | 0 | 35 | 100 % (37/37) |
| POS (>52 IU/mL) | 0 | 2 | 2 | |
| Total | 35 | 2 | 37 | |

**Table 4** Diagnostic concordance of TgAb in DTC subjects using the in-house reference intervals

| | Immunotech IRMA | | Total | Overall concordance |
|---|---|---|---|---|
| | NEG (≤30 IU/mL) | POS (>30 IU/mL) | | |
| Solid-phase radioassay | | | | |
| NEG (≤52 IU/mL) | 242 | 9 | 251 | 94.68 % (285/301) |
| POS (>52 IU/mL) | 7 | 43 | 50 | |
| Total | 249 | 52 | 301 | |
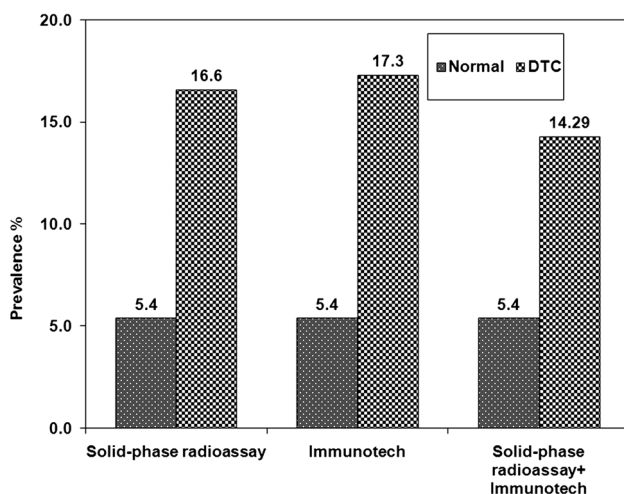


**Fig. 4** Percent prevalence of TgAb in normal population compared with that in patients with DTC using different methods (alone or in combination)

underestimation of Tg values. In view of this, estimation of TgAb in DTC patients is mandatory to help the clinicians to judge the true Tg values and hence the status of the patients. In the present study, we used in-house solid-phase radioassay to evaluate DTC patients for the presence of detectable TgAb and the incidence was found to be 17.3 % in comparison to 5.4 % in the control subjects. Thus, the relative risk of TgAb positivity in the DTC group compared to control group was more than two-fold. These findings are in agreement with other reports showing an increased prevalence of TgAb in patients with DTC compared with the general population [4–6, 14]. The above observed occurrence of TgAb (17.3 and 16.6 %) in DTC patients by us could be an underestimation, as higher levels of circulating Tg interfere in the measurement of TgAb [6]. Observation of the high diagnostic concordance in control group (100 %) compared to DTC (94.68 %) could be ascribed to the low incidence (5.4 %) of TgAbs in the control group resulting in a high random concordance [15]. Further, the sensitivities achieved by both in-house and commercial method were comparable. According to NACB recommendation, our reference interval study included 37 serum specimens from healthy, male subjects, in selecting whom, great care was taken to ensure that they did not have any of thyroid disorders. Of these, 2 (5.4 %) were screened for the presence of TgAb. This finding may be attributed to the presence of subclinical hypothyroidism with the co-existence of TgAb. A similar type of finding was reported by Sapin et al. [15].

TgAb is heterogeneous and the methods employed for the measurement of TgAb differ in their sensitivity and specificity and therefore, cannot be used interchangeably [4, 10, 14, 15]. The present study confirmed the other reports [14–16] wherein some samples showed the presence of TgAb by one method but not by another (Table 4). These differences were seen despite the use of same IRP standard, suggesting that TgAb with different epitope specificities (towards the immobilised Tg) were being recognized [4,10,15]. Although all the TgAb assays claim to be referenced to IRP 65/93, still there is a large degree of variation in the normal reference limits for TgAb. In the present study, the upper reference limits corresponding to

95th percentile were 30 and 52 IU/mL for anti-hTg IRMA (Immunotech) kit and solid-phase radioassay respectively, although both the assays used the same IRP standards. This difference in the threshold limit may be attributed to TgAb assays using preparations of h-Tg which, differed in their immunoreactivity towards the TgAb. Hence, for achieving better standardisation and minimum inter-method variation of TgAb assays, use of an universal standard preparation (International Reference Preparation) and identical antigen preparations for coating would be required. Apart from this, the heterogeneity of the antibodies present in patient's sample becomes an intrinsic factor responsible for such kind of discrepancy in the cut-off values which is inevitable. Nonetheless, it is essential that only the most sensitive immunoassay methods should be used to screen for the presence of TgAb. The sensitivity of the in-house solid-phase radioassay for the detection of TgAb was comparable to the commercial anti-hTg IRMA kit. Overall imprecision for both solid-phase radioassay and anti-hTg IRMA kit was ≤15 %.

In conclusion, the results obtained by us in sera from control as well as DTC group, by both the assays showed comparable performance. Stability of the Tg-coated tubes and longer shelf life of $^{125}$I-Protein A, gives the solid-phase assay a better option for routine estimation of TgAb in the patients serum samples. Furthermore, large discrepancies in the threshold levels mentioned by the different kit manufacturers indicate inter- method variation. Hence, for better clinical management, laboratories performing TgAb assays need to establish their cut-off reference limit for the type of assay adopted. Ideally, serial TgAb estimation in a patient should always be carried out in the same laboratory using the same method.

**Compliance with Ethical Standards**

**Conflict of interest** Chandrakala Gholve, J. Kumarasamy, Savita Kulkarni, and M G R. Rajan declares that the have no conflict of interest.

**Ethical Approval** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Informed Consent** Informed consent was obtained from all individual participants included in the study.

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