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Development of A Two-Step Immunoradiometric Assay for Serum Thyroglobulin Using A Combination of Polyclonal and Monoclonal Antibodies

KEYWORDS: Anti-thyroglobulin autoantibody, immunoradiometric assay, thyroglobulin

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ABSTRACT *Thyroglobulin (Tg) is used as a tumor marker in the follow-up cases of differentiated thyroid carcinoma (DTC) patients. A sensitive immunoradiometric assay (IRMA) for serum Tg estimation was developed at our Centre with analytical and functional sensitivities of 0.2 ng/ml and 1.0 ng/ml respectively. The assay used anti-Tg rabbit polyclonal antibody as capture antibody and 125I labeled monoclonal antibody as detector antibody. The assay was optimized for various reaction conditions with a working range of 1.0-800 ng/ml. The mean intra- and inter-assay coefficient of variation (CV) for QC samples was 6.95% and 8.85% respectively. Stability of the antibody coated tubes was up to 12 months. Good correlation was obtained when compared with commercial DiaSorin Tg IRMA kit (r=0.93, n=249, p<0.001)). All the samples included for the study were anti-Tg autoantibody (TgAb) negative as they are known to interfere in Tg assays.*

INTRODUCTION

Thyroglobulin (Tg) is a large globular glycoprotein, consisting of two symmetrical halves, with a molecular weight of 660 kDa (Edelhoch & Robbins, 1986). Serum Tg (s-Tg) is an established tumor marker for differentiated thyroid carcinoma (DTC) and is used in the management of patients with DTC (Shah et al., 1981; Spencer & Wang, 1995; Spencer et al., 1998). Several methods for the measurement of s-Tg are currently being used depending upon the requirement and available facilities at the institution. Tg can be measured by competitive immunoassay for e.g., radioimmunoassay (RIA) or by non-competitive sandwich immunoassay for e.g., immunoradiometric assay (IRMA). Due the incidence of autoantibodies to Tg in patients with DTC, the interpretation of s-Tg values will depend on the assay method used. Currently, commercial assays favor the IRMA format, because RIA suffers from the practical disadvantages of long incubation times, limited working ranges, short shelf-life and inherent instability of the tracer (^{125}I-Tg). Further, IRMA methods achieve better sensitivity than RIA, cover a wider working range, the labeled antibody reagent has a much better stability and shelf life. The non-isotopic format of IRMA viz., chemiluminescent immunometric assay (CLIA), has been widely automated and used in many commercial lab-analysers. Measurement of s-Tg remains technically challenging despite several methodological developments. Current Tg assays (RIA or IRMA) are still plagued by a number of technical problems, which influence the clinical reliability of s-Tg determinations. These include: lack of universal method standardization, inadequate assay sensitivity (especially with RIA) and imprecision; and interference from heterophilic antibody or Tg autoantibody (TgAb) that can cause under- or over-estimation in the assay. More than 10 years ago, efforts were made by the Community Bureau of Reference to develop an international Tg reference preparation, CRM 457. Most immunoassays are now calibrated directly or indirectly against this standard (Feldt-Rasmussen et al., 1996).

In the two-site IRMA system described here, Tg is captured onto anti-Tg rabbit polyclonal antibody (PAb) that is immobilized on a solid-phase. The detection of the captured Tg is by reacting it with ^{125}I labeled monoclonal antibody (^{125}I-MAb). The excess unreacted ^{125}I-MAb is removed by washing. The radioactivity from the antibody-antigen-antibody complex on the solid-phase is determined in a gamma counter. The concentration of Tg in the sample is directly proportional to the radioactivity and is determined by interpolation from the standard curve obtained with standards having known Tg concentrations.

At Radiation Medicine Centre, there is a large registry of over 10,000 thyroid cancer patients, who were treated with I-131 and come periodically for follow-up. About 4800 patient's sera are assayed annually for s-Tg by using commercial IRMA kits from different sources, depending on availability. To avoid method variability of kits from different sources, the present work was done to develop an in-house IRMA assay to ensure consistency in Tg assays over time. We used a combination of polyclonal and monoclonal antibodies, which were produced in-house in sufficient quantities.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents used were of analytical grade. PD-10 columns (pre-packed with Sephadex G-25) were obtained from Amersham Biosciences. Bovine serum albumin, Freunds' Complete and Incomplete Adjuvants, were obtained from Sigma Chemical Company, St. Louis, USA. Carrier free Na^{125}I was supplied by Radiopharmaceuticals Division, BARC, Trombay, Mumbai. Mouse anti-Tg monoclonal antibody were produced and provided by one of the authors (AAD). Glutaraldehyde solution (25%) was ob-

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tained from M/s S.D. Fine Chemicals, Mumbai. Star polystyrene RIA tubes (70mm X 10mm) were procured from M/s Tarsons India Ltd. Calcutta. Comparative validation studies were carried out using commercial IRMA assay kits (Tg-CTK) from DiaSorin, Saluggia, Italy. The remaining chemicals of AR or other suitable grade of purity were purchased locally.

Buffers and solutions

1. Phosphate buffer (PB): 0.025 M phosphate, pH 7.5 containing 0.01% sodium azide.
2. Phosphate Buffered Saline (PBS): PB with 0.15 M sodium chloride.
3. PBS-EDTA: PBS containing 0.01 M EDTA (assay buffer).
4. Bicarbonate buffer: 0.1 M sodium bicarbonate, pH 8.6.
5. Borate buffer: 0.1 M boric acid, pH 8.0.
6. Tris-buffer: 0.1 M Tris buffer, pH 8.2 containing 0.02 M EDTA and 0.005% Thiomersal.
7. Wash buffer: PBS with 0.05% Tween 20.

Instruments and Software

1. RIASTAR Multi-well gamma counter from Packard, USA was used for counting and plotting dose-response curves.
2. RIA data-analysis was performed using WHO-Program for RIA written by Prof. Ray Edwards, Dept of Molecular Endocrinology, Middlesex Hosp Med School, London, U.K.

Preparation of working Tg standards

Tg was purified from thyroid tissue of human origin. Thyroid tissue (from three thyroid glands) was collected at 4-6 h of autopsy. Tg from these thyroid tissues was isolated according to the method of Mouriz & Stanbury, 1968, and further purified by exclusion chromatography. To use as standards in IRMA, the Tg preparation was diluted in human Tg free serum (stock I containing 100 µg/mL of Tg and stock II containing 10 µg/mL of Tg). These stocks were aliquoted and stored at -20°C. Various Tg concentrations to be used as Tg standards, were prepared from stock II by diluting appropriately with PBS-EDTA buffer. The in-house prepared Tg standards were checked by comparing the dose response curves for in-house standards and DiaSorin kit standards. DiaSorin kit standards are calibrated against Tg reference preparation CRM 457 (Feldt-Rasmussen et al., 1996).

Human Tg free serum

Serum samples from disease free patients of DTC, who had undergone thyroidectomy, and/or radioiodine ablation therapy with undetectable s-Tg and endogenous TgAb levels were pooled and used as Tg free serum for preparing standards.

Collection of Normal Rabbit Serum (NRS)

NRS was collected from non-immunized New Zealand white rabbits and stored at -20°C.

Production of rabbit anti-hTg polyclonal antibody

Antibodies to human Tg were raised in 3 normal rabbits by subcutaneous immunization with 400-500 µg of purified Tg in Freunds' Complete Adjuvant. Following the initial immunization, 3-4 booster immunizations with the same amount of Tg in Freunds' Incomplete adjuvant was administered at 2-4 weeks intervals. The rabbits were then bled at fortnightly intervals, after the final booster and the sera obtained were tested for antibody by Ouchterlony double immunodiffusion against purified Tg, thyroid extract and normal serum. All the rabbits responded well after four boosters, and their antisera gave a single intense precipitin line with purified Tg and thyroid extract. However, no visible precipitin line was seen with normal human serum. The antisera was aliquoted and stored at -20°C till further use. The animals were periodically boosted and blood collected. Antisera production was with the approval from BARC Animal Ethics Committee.

Immobilization of polyclonal anti-Tg on polystyrene solid-phase

'Star' polystyrene tubes were chemically activated by adding 1 mL of 1.5% glutaraldehyde solution (v/v) in 0.1 M NaHCO3 buffer, pH 8.6, and left overnight at room temperature. The glutaraldehyde solution was then aspirated out and all the tubes were washed twice with 2 mL of 0.1 M NaHCO3 buffer. These tubes were coated with 50 µg of Ig fraction of PAb in 0.3 mL of 0.1 M borate buffer, pH 8.0, and again incubated overnight. The unbound PAb solution was aspirated and the tubes were washed twice with 2 mL of 0.1 M NaHCO3 buffer. The unreacted glutaraldehyde activated 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 again blocked but this time 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 to 4 hours and stored in airtight zip sealed polyethylene bags at 4°C.

Radioiodination of monoclonal anti-Tg antibody

Anti-Tg MAb was labeled with ^{125}I by the Iodogen method (Fraker & Speck, 1978). Radioiodination was carried out using 5 µg of MAb and 3 – 4 MBq of ^{125}I. The reaction was allowed to proceed for 4 minutes. The unreacted ^{125}I was separated from the labeled protein by gel-filtration on a PD-10 column and eluted with PBS-EDTA buffer. Two distinct peaks containing radioactivity were obtained, the first corresponding to the labeled antibody and the second to free iodide. The tubes containing higher amounts of radioactivity from the first peak were diluted with PBS-EDTA buffer with 2.5% BSA, aliquoted and stored at a temperature of -20°C, till further use in IRMA. The labeling efficiency and the specific activity of the ^{125}I-MAb were calculated.

Optimization of assay

Using the ^{125}I-MAb, various reaction parameters such as concentration of reagents, reaction kinetics, sequential addition of the reagents, time and temperature were evaluated to arrive at a suitable assay system which are given in Table 1.

TABLE 1 Optimized Assay Parameters for In-house s-Tg IRMA

Parameter	Optimized Parameter
Concentration of capture antibody (PAb)	50 µg/tube
Coating volume	300 µL
Standard/sample volume	100 µL
Buffer (in standard/sample)	100/200 µL
Hormone free serum in standards	100 µl
Concentration of detector antibody	4800 Bq/300 µL
Incubation Period	

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a. Primary	15 hrs.
b. Secondary	3 hrs.

To PAb coated 'star' tubes, following components were added in sequence:

1. 100 µL standard Tg (diluted in assay buffer) or 100 µL of serum sample /QC sample.
2. 100 µL Tg free serum (NRS) in tubes containing standard Tg.
3. The final volume was made to 300 µL with assay buffer.
4. The tubes were incubated on orbital shaker for overnight (15 h) at room temperature.
5. Content of the tubes was aspirated and washed 3 times with 3 mL of wash solution.
6. 300 µL of ^{125}I-MAb (4800 Bq) was added and further incubated for 3 h on orbital shaker at room temperature.
7. Washing step was repeated and the tubes were blotted and counted for radioactivity.

Quality control tests like non-specific binding, precision, sensitivity, reproducibility, recovery, and parallelism test were performed to evaluate the developed IRMA system. With 20% CV as the acceptable assay precision, the working range was determined from the precision profile. The high dose 'Hook-Effect' was studied by developing a standard curve with a high Tg concentration upto 25,600 ng/ml. Also, the stability of the coated tubes (stored at 4°C) was checked over a period of 12 months.

Measurement and comparison of s-Tg levels

Serum samples from patients referred to our Centre (n=249), with an established diagnosis of DTC were assayed for s-Tg. Serum samples were stored at 4°C until assayed the same day or stored at -20°C until the next assay. Developed s-Tg IRMA was compared with the commercial DiaSorin Tg IRMA kit and linear regression analysis was performed for the data obtained.

RESULTS

Evaluation of Tg standards

The dose-response curves of the in-house prepared Tg standards were compared with that of the DiaSorin kit standards (Figure 1). The standard curves were comparable and almost superimposable.

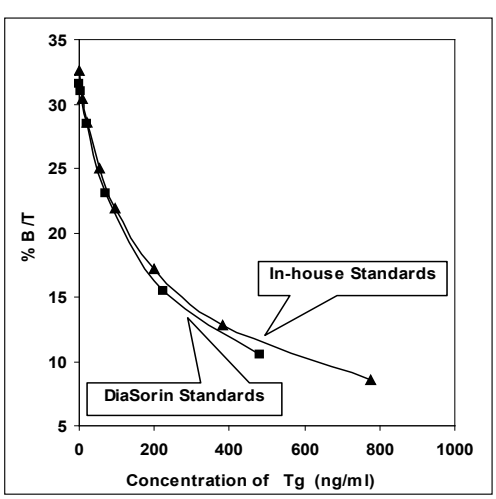

FIGURE 1 Comparison of dose response curves for in-house and DiaSorin kit standards for Tg

Labeling efficiency and specific activity of anti-Tg ^{125}I-MAb

Figure 2 shows two distinct peaks (containing radioactivity) obtained after radio-iodination of anti-Tg MAb. The first peak corresponded to the ^{125}I-MAb and the second to free iodide. The efficiency of labeling obtained at five different radio-iodination procedures ranged from 70-85%. The specific activity of ^{125}I-MAb varied from 444-703 kBq/µg.

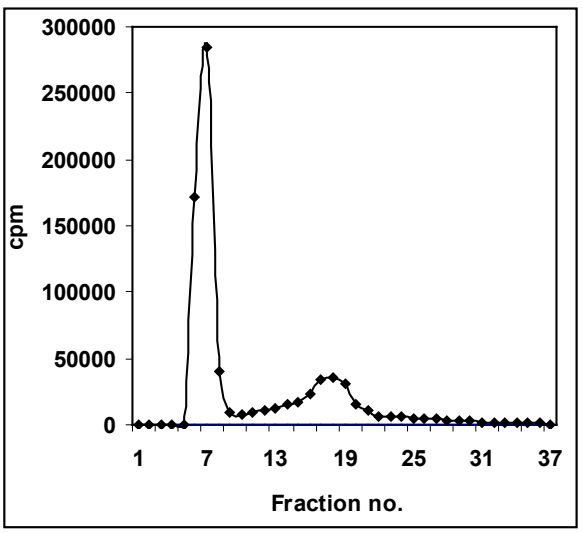

FIGURE 2 Radioiodination of anti-Tg murine monoclonal antibody

Characteristics of the assay

For calculating the analytical sensitivity of the assay, ten 'zero' Tg standard (B0) tubes were processed in a single assay along with the other Tg standards. Mean and standard deviation (SD) was calculated for the counts of ten B0 tubes. Dose-response curve was constructed on linear graph paper with mean response values. Theoretical sensitivity for IRMA was calculated at B0 binding with 99% confidence level (Mean B0+3SD) which was 0.2 ng/ml. The clinically useful range was found to be 1 – 800 ng/mL and this generally represents that concentration range where the imprecision of the assay is less than 20%.The assay had a functional sensitivity of 1.0 ng/ml which was determined from assays of 4-6 months.

Replicates of two quality control samples (QC1=2.6±0.3 ng/mL & QC2=42± 4 ng/mL) were setup in a single assay as well as in assays carried out at different intervals to characterize the intra- and inter-assay precision. Analytical recovery varied between 85-110% when carried out by adding known amount of Tg (25 and 100 ng/mL) to two serum samples, negative for the presence of TgAb. Parallelism of the assay was evaluated by assaying a serum sample (with high Tg content) serially diluted with Tg free serum. The observed concentration to the expected ranged from 82.7% to 90.6%. With 20% CV as the acceptable assay precision, the working range was determined as 1.0-800 ng/ml. Since our IRMA follows a 'two-step' assay design, it did not show any 'Hook-Effect' up to a concentration of 12,800 ng/mL. Anti-Tg coated tubes stored as described above over a period of 12 months were found to have acceptable specific binding ranging between 33 to 22% with a NSB of 0.28 to 0.47% **(Table 2).**

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TABLE 2 Characteristics of In-house IRMA for s-Tg

Parameter	s-Tg IRMA
Sensitivity	
a. Analytical	0.2 ng/mL
b. Functional	1.0 ng/mL
NSB	0.28 to 0.47%.
Precision	
a.Intra-assay variation	Control A: 2.52 ± 0.20 ng/mL, %CV=7.9
	Control B: 40 ± 2.4 ng/mL , %CV=6.0
b. Inter-assay variation	Control A: 2.6 ± 0.25 ng/mL, %CV=9.6
	Control B: 42 ± 3.4 ng/mL , %CV=8.1
Dilution test	82.7 - 90.6%
Recovery	85 - 110%
Assay range	1.0-800 ng/mL
Hook effect	No hook effect up to 12,800 ng/mL
Stability of the coated tubes	12 months
Stability of the tracer	2 months
Total incubation time	18 hrs

Comparison of s-Tg levels in DTC patients

Comparison of serum Tg levels in the serum samples of the patients with DTC, estimated by our IRMA method and by the IRMA kit from DiaSorin, showed good agreement. Regression analysis showed good correlation (r=0.93, n=249) as shown in Figure 3. Tg autoantibody positive samples were excluded from the analysis as they are known to interfere in Tg assays.

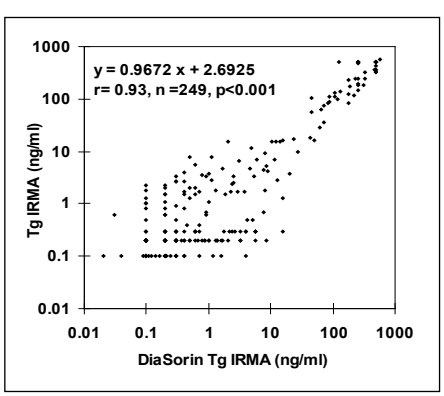

FIGURE 3 Scatter diagram for Tg by in-house developed IRMA and commercial Tg IRMA kit (DiaSorin) in TgAb negative samples (n=249)

DISCUSSION

At our Centre, a conscious decision was made to switch over using an in-house RIA to commercial IRMA in view of the increasing number of samples received for serum Tg estimation. We then decided to develop an in-house Tg IRMA to avoid assay variability and bias inherent in different batches of commercial kits. All the reagents for the assay were prepared in-house viz. hTg and anti-Tg antibodies (PAb and MAb).

Data from a multicenter study showed that the inter laboratory variability in samples without TgAb was abnormally high with %CV varying between 48%-120%. Also the within-methods and between-laboratories CVs were unsatisfactory (49%-117%). In samples containing TgAb the CV increased up to 93%-116% due to different degree of interference from TgAb in different methods. These systemic differences between methods were mainly due to both different calibrators and different specificities of the antibodies utilized by the users (Iervasi et al., 2006). In order to minimize these differences, CRM 457 is being adopted either directly or indirectly for the calibration of Tg immunoassays by most of the laboratories. Even then, serum Tg levels determined by methods using this standard vary by as much as fourfold (Baloch et al., 2003). Inter-method differences are likely to remain; hence, particular care should be taken when comparing s-Tg values that are obtained by using different immunoassay systems.

Recovery and dilution tests were performed to ensure that the calibration is accurate and that the matrix used for making standards is appropriate. The bias between different Tg methods may also result from differences between the Tg-free matrix used to dilute standards and patient serum. Ideally, the diluent used for preparing standards should be Tg-free/TgAb-free human serum or alternatively, a non-serum matrix that has been selected to produce a signal (radioactive counts, relative light units etc) identical to Tg-free/TgAb-free human serum. Due to inadequate availability of the human hormone free serum, the IRMA assay was optimized and standardized using NRS which yielded similar binding and non-specific binding results when compared with human Tg free serum.

For long term follow-up of cancer patients, it is strongly recommended that serial Tg measurements in a patient be made in the same laboratory using the same assay method because Tg values are not interchangeable among laboratories, even when using the international Tg reference standard which reduces but does not eliminate biases between Tg methods (Spencer, 1996; Mazzaferri et al., 2003; Harish, 2006).

The in-house developed IRMA for serum Tg offers required assay sensitivity that is needed for clinical decision making and hence, can be used for routine estimation of serum Tg.

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