Biochemical Tumour Marker

Development of Immunoradiometric Assay Kit for Measuring Serum Thyroglobulin in Differentiated Thyroid Cancer Patients

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RMC-Tg kit (deployed as biochemical tumour marker) developed in Radiation Medicine Centre

ABSTRACT

Thyroglobulin (Tg) serves as a useful biochemical tumour marker in follow-up cases of patients with differentiated thyroid carcinoma (DTC). A sensitive immunoradiometric assay (IRMA) for serum thyroglobulin (Tg) estimation was developed at Radiation Medicine Centre, with analytical and functional sensitivities of 0.10 ng/ml and 0.4 ng/ml, respectively. The assay uses anti-Tg camel polyclonal antibody as capture antibody and ^{125}I labeled monoclonal anti-Tg antibody as detector antibody. A good correlation was obtained on comparison with commercial Izotop® TG IRMA kit (n=142, r=0.98, p<0.001). So far, we have analyzed approximately 36,000 routine clinical samples with the new in-house developed kit. Indigenously developed radioisotopic TG IRMA kit (RMC-Tg KIT) is convenient, robust, cost-effective, automated and is being used satisfactorily for the last 8 years, in the routine *in-vitro* 'Immunoassay Services' at RMC by substituting the expensive commercial Tg kits.

KEYWORDS: *Thyroglobulin, Camel polyclonal antibody, Differentiated thyroid carcinoma, Immunoradiometric assay, Monoclonal antibody, Sensitivity.*

Introduction

Radiation Medicine Centre (RMC), a Nuclear Medicine Department of Bhabha Atomic Research Centre, is one of the largest referral bases in India for the management of patients with various thyroid disorders including differentiated thyroid cancer (DTC)[1]. For the better management of patient services, various developments; including in-vivo and *in-vitro* diagnostics, and therapeutic strategies are continuously being explored and applied here. RMC being a pioneer in Nuclear Medicine is equipped with the infrastructure for the development and use of radioisotopic kits for routine in-vitro patient services. Various assay formats for serum thyroglobulin (Tg) and thyroid autoantibodies *viz.*, anti-Tg autoantibody (TgAb) and anti-thyroid peroxidase autoantibody (TPOAb) have been developed and validated at RMC[2-6]. Present work herewith also endeavors to fulfill the prerequisite by using the in-house developed TG IRMA kits (RMC-Tg KIT) for *in-vitro* measurement of Tg in DTC patients.

Patients diagnosed with DTC following thyroidectomy undergo radioiodine (^{131}I) therapy for ablation of residual thyroid tissue and treatment of recurrent disease and metastases[7]. Serum Tg is an established tumour marker used in the management of DTC patients. Several methods for the measurement of serum Tg are being used depending upon the prerequisite and infrastructure available at the institution. The type of method, radioimmunoassay (RIA) or immunometric assay (IMA), used to measure Tg in sera, governs the clinical utility and interpretation of Tg values. Currently, commercial assays favor the IMA technique, because RIA poses certain technical limitations like long incubation times, limited working ranges, short shelf-life and inherent instability of the tracer (^{125}I-Tg). In contrast, IMA methods achieve better sensitivity than RIA in a shorter incubation time, cover a broader working range, use a mores

labeled antibody preparation and in their non-radioisotopic format, *viz;* chemiluminescent immunometric assay (CLIA) have the potential to be automated. However, non-radioisotopic assays like CLIA and Fluoroimmunoassay have a high background (due to autofluorescence of biological samples and/or interfering compounds) and high instrumentation cost. Today even after radical advancement in the immunoassay methodologies, measurement of Tg still remains technically challenging, thereby influencing the clinical reliability of Tg determinations. Lack of universal method standardization, inadequate assay sensitivity (especially with RIA) and precision; and interference from heterophilic antibody or Tg autoantibody (TgAb) that can cause under or overestimation in Tg concentration are the key factors responsible for the reliability of current Tg assays[8]. However, due to the occurrence of TgAb in approximately 25% of DTC patients; its detection and evaluation of its interference during serum Tg measurement becomes mandatory [8]. Community Bureau of Reference has developed an international Tg reference preparation, CRM 457, which is being used directly or indirectly for calibration in most immunoassays[3, 8].

The development of a two-step immunoradiometric assay (IRMA) using rabbit anti-Tg polyclonal antibodies has been discussed earlier[3]. The development of IRMA for routine

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clinical use in patients demanded a large and uninterrupted supply of antibodies, and producing IRMA kits using commercial antibodies was also not economically feasible. Given this, present efforts were directed to develop an IRMA system that uses anti-Tg polyclonal antibodies from a larger species like a camel. In this collaborative work, anti-Tg polyclonal antibodies were raised in camels at ICAR (Indian Council of Agricultural Research)-National Research Centre on Camel, Bikaner. In the one-site IRMA system mentioned here, Tg is captured onto anti-Tg camel polyclonal antibody (PAb) bound or immobilized on a solid-phase (polystyrene tubes). The assay is carried out by reacting ^{125}I labeled monoclonal anti-Tg antibody (^{125}I-MAb) with Tg, which is bound to the PAb on the solid phase. Excess of unreacted ^{125}I-MAb is removed by washing the tubes with the wash buffer. The sandwich formed remains bound to the solid-phase antibody which is later quantified by counting the bound radioactivity in a gamma counter. The concentration of Tg in samples is directly proportional to the radioactivity and is obtained by interpolation from the standard curve.

Thyroglobulin autoantibody, heterophile antibodies, or non-specific effects in a patient's serum can interfere with serum Tg measurement thereby giving underestimated or overestimated Tg values depending upon the type of assay used[8]. Nevertheless, the presence of TgAb leads to underestimation of the Tg concentration in the IRMA system which can be detected by performing parallelly the 'Tg-Recovery Test' for each sample under investigation, using RMC-Tg KIT.

RMC has a large registry of more than 15,000 DTC patients who are monitored for the efficacy of treatment during their routine follow-up. Management of these patients is based on serum Tg measurement and ^{131}I whole-body scanning. Hence, our aim was to develop a low-cost in-house TG IRMA kit for the estimation of serum Tg in these patients; as an import substitute for high-cost commercial IRMA kits. This article reports the development of single-step IRMA for serum Tg measurement for routine clinical use in DTC patients. The flow chart in Fig.1 illustrates various steps involved in the development of RMC-Tg KIT. About 5800 patient's sera are quantitated annually for Tg and Tg-Recovery using the indigenously developed kits. Our end-users are patients from RMC OPD, Tata Memorial Hospital, Rajiv Gandhi Cancer Institute and Research Centre, New Delhi; and a few nearby Government hospitals (Fig.2). In the past 8 years, we have produced approx. 1702 kits (100 determinations/kit) and have investigated around 36,000 routine serum samples for Tg and 'Tg-Recovery Test'.

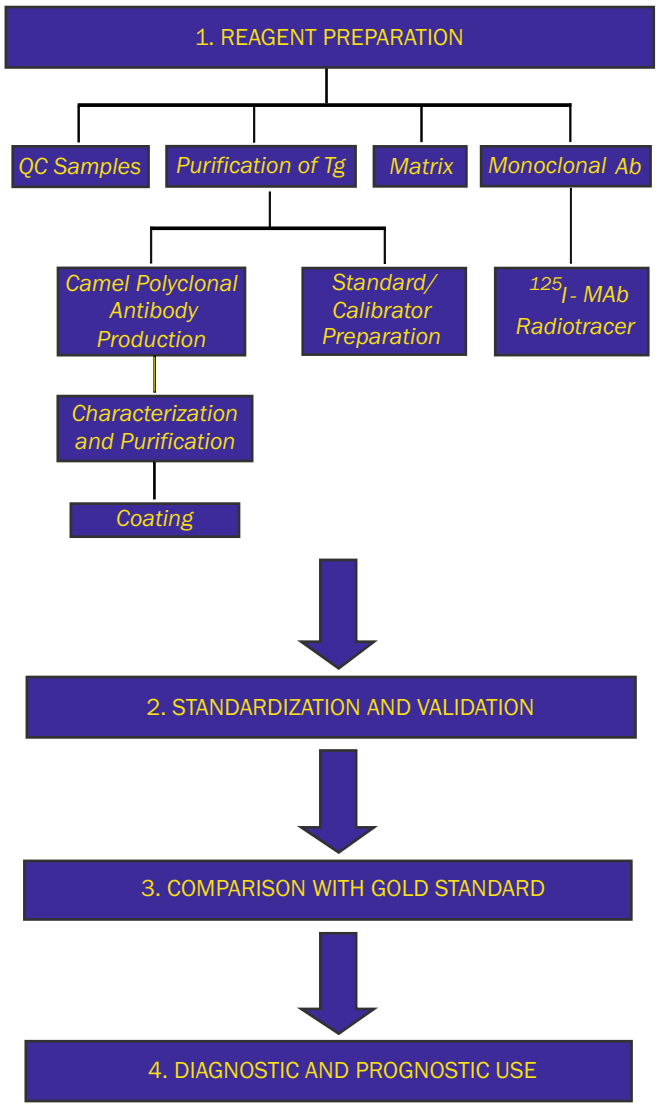

Fig.1: Steps involved in the development of RMC-Tg KIT.

Important Highlights of the Work

1. Reagent preparation in bulk for the production of indigenous RMC-Tg KITs.
2. Standardization and Validation of the in-house developed Tg IRMA assay.
3. Comparison of in-house Tg IRMA assay with a gold standard method.
4. Application of RMC-Tg KITs for the management of DTC patients at RMC.
5. Novel components of the RMC-Tg KIT influencing the total production cost.
6. Retrenchment in the Organizational Annual Budget by replacing Commercial TG Izotop® kit with cost-effective in-house developed RMC-Tg KIT.

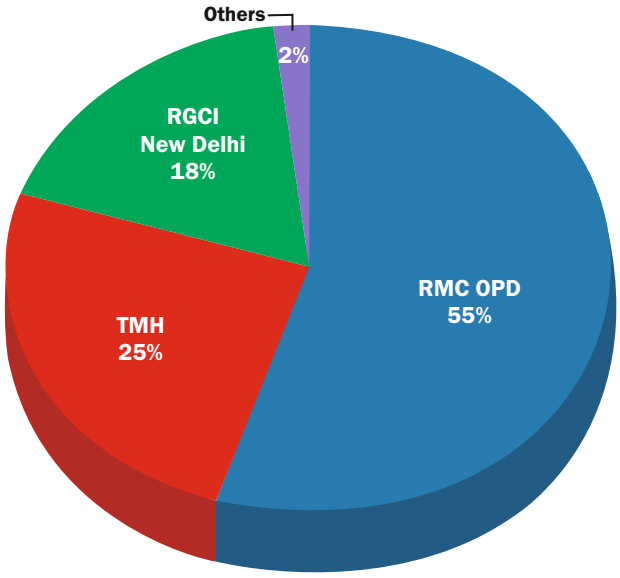

Fig.2: End-users of the indigenously developed RMC-Tg KIT and their break-up.

Experimental

1. Reagent Preparation

I. *Extraction and purification of human Tg (antigen preparation)*

The in-house purified Tg from normal human thyroid tissues was extracted within 4-6 h of the autopsy, purified and used for standard preparation and evaluation by comparing the dose-response curves with Izotop® kit standards. Various steps followed in the purification of Tg are illustrated in Fig.3. Standards with varying Tg concentrations were prepared using different matrices: synthetically prepared in-house matrix, human hormone-free serum and pooled Tg serum samples from disease-free DTC patients. Tg levels assayed using these matrices were compared with commercial kit (Izotop® TG IRMA kit) results. The Izotop® kit standards were calibrated against CRM 457. The stocks of purified Tg (preserved in phosphate-buffered saline) are maintained at -30 °C at RMC.

ii. Production of camel anti-hTg polyclonal antibody

Antibodies to human Tg were raised in 2 young male Indian Camels (C. dromedarius, J242 and J244) by subcutaneous immunization with 400 µg of in-house purified Tg in Complete Freund's Adjuvant. Following the initial dose, 3-4 booster doses with the same amount of Tg in Incomplete Freund's adjuvant were administered after 3-4 weeks respectively. The camels were then bled at fortnightly intervals after the final booster. Antisera were characterized for their specificity, avidity and titre; and stored at -20 °C till further use.

iii. Immobilization of camel polyclonal anti-Tg on polystyrene solid-phase

By passive adsorption, the polystyrene tubes were immobilized with approximately 50 µg of caprylic acid fractionated [9] or ammonium sulfate precipitated camel Ig fraction of PAb (in 0.3 ml of 0.1 M borate buffer, pH 8.0) and incubated overnight at room temperature. The unbound PAb solution was aspirated and the tubes were washed twice with 2 ml of 0.1 M $NaHCO_3$ buffer. The empty sites on the polystyrene surface were blocked with 0.5 ml of 0.1 M Tris buffer containing 0.3% BSA (w/v) and 1% sucrose (w/v) for an overnight period. The blocking solution was aspirated and the tubes were air-dried for an overnight period and stored in airtight plastic zip sealed bags at 4 °C for further use. The entire antibody coating process which is a labor-intensive step is performed manually. Antibody pipetting is done manually using Eppendorf-Multipette whereas aspiration and washing are performed using an in-house fabricated aspirator and washer.

iv. Radioiodination of monoclonal anti-Tg antibody

In-house produced murine anti-Tg monoclonal antibody (MAb) [10] was labeled with $Na^{125}I$ by iodogen method following radioiodination protocol mentioned in our earlier report[3].

2. Standardization and Validation of Tg assay

Various reaction parameters such as concentration of reagents, reaction kinetics, sequential addition of reagents, time, temperature, etc. were evaluated to arrive at a suitable assay system which is given in Table 1.

Sensitivity, non-specific binding (NSB), precision, reproducibility, recovery, and dilution test were some of the quality control parameters performed to evaluate the performance of indigenous Tg IRMA assay. Considering 20% coefficient of variation (CV) as the acceptable assay precision, the working range was determined from the precision profile. The assay was studied for the influence of excess Tg concentration leading to high dose 'Hook-Effect'. The stability of the capture antibody (antibody-coated tubes stored at 4 °C) was established by checking the immunoreactivity of the antibody-coated tubes for 24 months.

Table 1: Optimized parameters of Tg assay.

Parameter	Optimized values
Concentration of capture antibody (PAb)	~ 50 µg/tube
Coating volume	300 µl
Standard/sample volume	100 µl
Concentration of tracer antibody 125 I-MAb	4800 Bq/200 µl
Incubation Period	15-16 hrs

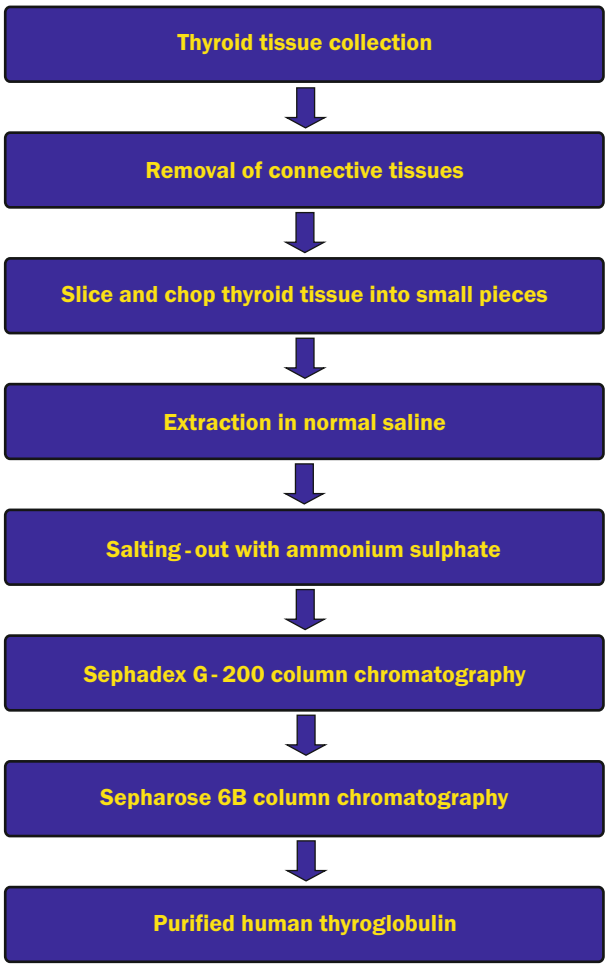

Fig.3: Extraction and purification of thyroglobulin obtained from human thyroid tissue.

Tg IRMA protocol

To the antibody-coated tubes, the following components were added in sequence:

- 100 µl standard Tg, QC or serum sample.
- 200 µl of ^{125}I-MAb (4800 Bq).
- Incubate the tubes overnight (optimized for 15-16 h) on an orbital shaker at room temperature.
- Aspirate the content of tubes and wash twice with 2 ml of wash solution (PBS with 0.05% Tween 20).
- Decant the fluid and blot on filter paper and count the tubes for radioactivity in RIASTAR Multi-well gamma counter / SR 300 RIA autoanalyzer.

Table 2: Overview of Cost and total number of RMC-Tg KIT(s) produced and utilized in RMC.

	Overview of RMC-Tg KIT production	
1.	RMC-Tg KITS produced Since 2014	1702 (170200 Tubes)
2.	Samples analysed Since 2014	72000 Determinations
	Tg *Tg Recovery test*	36000 36000
3.	**Cost (in Rupees)/Kit (100 Determinations)**	
	Commercial (IZOTOP Kit)	~17,500
	RMC-Tg KIT	~2500
4.	**Annual Cost of TG Kits (in Rupees)**	
	Commercial (IZOTOP Kit)	~40,00,000
	RMC-Tg KIT	~5,50,000

Tg-Recovery Test

The concentration of the recovery serum (approximately 500 ng Tg/ml) is checked with serum diluent or diluent recovery (DR) reference tubes. Recovery (in %) in each serum sample is then calculated using the following formula [2].

Recovery (in %) in the serum sample:

$$\frac{\text{ngTg/ml } R_x - \text{ng Tg/ml } S_x}{\text{ng Tg/ml DR}} \times 100 = \%\ \text{Recovery}$$

Where Rx is recovery obtained, Sx is the Tg concentration for the sample and DR is the recovery obtained with serum diluent (to check the concentration of recovery serum).

3. Comparison of in-house Tg Assay with a Gold Standard Method

Serum samples from patients referred to Radiation Medicine Centre, with an established diagnosis of DTC (n=142) were analyzed for the presence of serum thyroglobulin. Developed serum RMC-Tg KIT was validated and compared with a gold standard and commercially available TG Izotop® kit. A linear regression analysis was performed for the data obtained.

Results and Discussions

Serum Tg measurement is a technically challenging assay for a marker of choice during the follow-up of DTC patients. Hence, the user of Tg assay should be well-versed about the technical difficulties. At our Centre, because of an increasing number of samples for serum Tg estimation, we developed TG IRMA wherein all the reagents are prepared in-house *viz*., hTg, anti-Tg antibodies (PAb and MAb), tracer, matrix, standards and QC samples. One of the principal reagents involved in the manufacture of TG kits is purified Tg from human thyroid tissue. Commercially available human Tg is highly expensive. Therefore, at RMC, we have extracted and purified approximately 1200 mg of Tg (total Tg yield) from two thyroid tissues which is being used for immunization, standard preparation and radiolabeling (for testing the antibody titre). These purified Tg stocks are appropriately stored at RMC and apart from its main use in RMC-Tg KIT production had other applications too [11-13].

The uniqueness of the in-house developed Tg assay lies in the application of camel polyclonal antibodies for immobilization as a capture antibody. Conventionally immunometric assays for Tg uses a panel of two or more monoclonal antibodies for capture or signal. Nonetheless, the use of monoclonal antibodies has a limitation of Tg epitope specificities in detecting abnormal tumor Tg isoforms[14]. Secondly, in comparison to rabbits [3], camel provides a large supply of polyclonal antibodies, which is one of the prerequisites during the development of any non-competitive immunoassays. Thus, the preparation of regents in bulk for the production of RMC-Tg KIT has greatly reduced the overall cost in comparison to the highly expensive imported TG IRMA kits (Table 2), without compromising on the quality of the performance. In addition to this, by adopting a passive adsorption method of coating as compared to chemical means [3] there is a reduction in the overall time taken and the manpower involved in the antibody immobilization process; a contributing aspect in reducing the cost of the kit. Initially, we had used the ammonium sulfate precipitation method as it is the most commonly used method. But later switched over to the caprylic acid method as it yields a high amount of purified IgG without reducing antibody titre, immunoreactivity, and distorting its structure. The stability of the capture antibody (camel anti-Tg coated tubes stored at 4 °C), was checked for 24 months which showed specific binding ranging between 35 to 45% and NSB between 0.10 to 0.25%. Therefore, even the 24-month-old antibody-coated tubes could be used satisfactorily for the estimation of Tg.

Variations between different methods used for assaying serum Tg arises from differences between the matrix used to prepare standards and dilute patient's serum. We have prepared a well-defined stable matrix, synthetic hormone-free serum (SFS) and determined its utility as a stripped-out serum substitute in assays for serum Tg. This allows the production of the matrix in bulk quantities, with precise lot-to-lot consistency. Additional benefits include the complete absence of endogenous Tg, interfering antibodies, and potentially harmful bloodborne pathogens. The in-house prepared Tg standards were evaluated by comparing dose-response curves for in-house standards and Izotop kit standards. Standard curves were found to be almost superimposable (Fig.4) indicating that it can be used for Tg IRMA assays for clinical application.

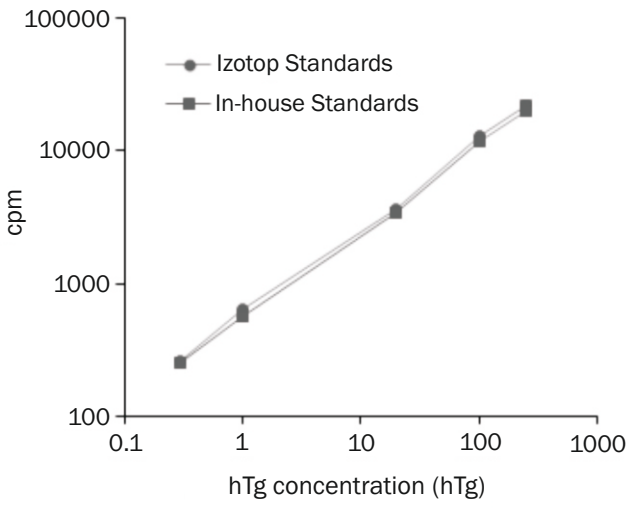

Fig.4: Comparison of dose response curves for in-house and Izotop® kit standards.

Recovery and dilution tests were also performed to ensure the accuracy and suitability of the matrix used for making standards. However, in our laboratory for routine usage, RMC-Tg KIT(s) are being produced using the leftover (excess) TSH standards (prepared in bovine serum) from Immunotech, France, after further appropriate modification and testing (viscosity of the matrix is compared with that of the SFS and dose-response curves are compared); as a basis of the matrix for preparing Tg standards. This has tremendously helped us in making in-house produced RMC-Tg KIT more economical.

Fig.5 shows two distinct peaks obtained after labeling anti-Tg monoclonal antibodies. The first peak corresponds to radiolabeled antibody and the second to free iodide. The labeling efficiency and specific activity ranged from 70-85% and 444-703 kBq/µg respectively. Sensitivity and NSB for IRMA assays are governed by two important parameters *viz*; specific activity and quality of the labeled antibody. The assay has been well optimized for radioiodination procedure to yield an assay with analytical and functional sensitivities of 0.10 ng/ml and 0.4 ng/ml respectively, and NSB < 0.2%.

Intra-and-inter-assay precision was respectively determined by setting-up replicates of two quality control samples in a single assay as well as in assays carried out at different intervals. Analytical recovery, estimated by adding a known amount of Tg to two serum samples, negative for the presence of TgAb, ranged from 78% to 120%. Parallelism of the assay was evaluated by assaying a serum sample, with high Tg content, serially diluted using matrix. The observed concentration to the expected (% O/E) ranged from 82% to 120% displaying a good agreement between the two. With 20% CV as the acceptable assay precision, the working range was determined as 0.4-300 ng/ml. TG IRMA which is an 'inclusive-assay' did not show any kind of high dose 'hook-effect' up to a concentration of ~12,800 ng/ml. The salient features of the indigenous RMC-Tg KIT are listed in Table 3.

Laboratories can implement one of the steps to identify TgAb interferences; measurement of TgAb, discordance between Tg values by RIAs and immunometric assays; and recovery of added Tg[7]. We used the 'Tg-Recovery Test' for detecting the interference of anti-Tg autoantibodies in the Tg IRMA system. Recoveries obtained between 80% and 130% are considered valid, levels of <70% or >130% are due to interference (autoantibody interference and non-specific serum interference) and the Tg level of the relevant original sample is considered invalid. Levels between 70%-80% are due to equivocal autoantibody interference. Tg Recovery (%) in

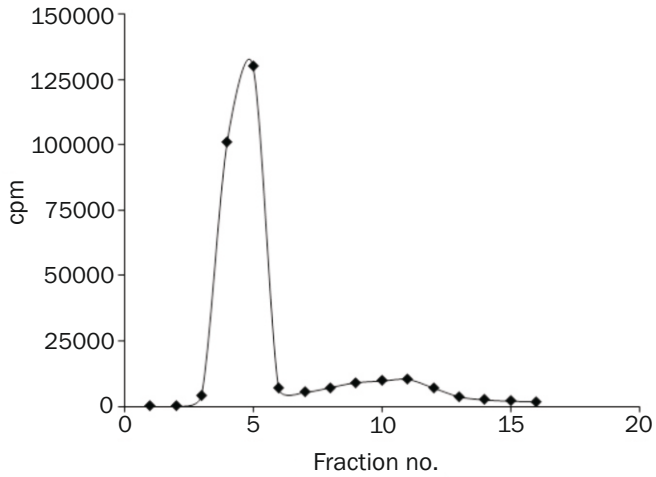

Fig.5: Elution profile of ^{125}I-monoclonal anti-Tg antibody post radiolabeling.

Table 3: Salient features of RMC-Tg KIT.

Parameter	TG IRMA
Sensitivity	
a. Analytical *b. Functional*	0.10 ng/ml 0.40 ng/ml
NSB	< 0.2%
Precision	
a. Intra-assay variation	Control A: 0.9 ± 0.09 ng/ml, % CV=9.9 Control B: 73.9 ± 5.8 ng/ml, % CV=7.8
b. Inter- assay variation	Control A: 0.95 ± 0.13 ng/ml, % CV=14.4 Control B: 77.8 ± 8.9 ng/ml, % CV=11.5
Recovery	78 to 120%
Linearity	82 to 120
Assay range	0.1-300 ng ml
Hook effect	No hook effect up to 12,800 ng/ml
Stability of antibody coated tubes	24 months
Stability of tracer	2 months
Incubation time	15-16 hrs.

complement with Tg IRMA information aids in recognition of interference in Tg measurements[15].

Comparison of Tg levels in the serum sample of patients with DTC, estimated by the present method and by Izotop TG IRMA kit, showed good agreement. Regression analysis showed good correlation (r=0.98, n=142, p<0.001) as shown in Fig.6. TgAb positive samples were excluded from the analysis as they are known to interfere in all Tg assays. Use of different sources and nature of kit calibrators, heterogeneity of analyte, antibodies with different specificities towards the Tg isoforms are all responsible for variations in Tg values obtained using different methods. Most of the Tg assays use CRM 457 for calibration, still, inter-method variability exists. Therefore, as recommended by American Thyroid Association, the same immunoassay should be used while monitoring an individual patient [16].

Conclusion

This publication addresses the preparation of principal reagents and steps involved in the production of indigenous RMC-Tg KIT (Fig.7) at the laboratory level for monitoring of serum Tg levels which is of great clinical significance in the long-term management to detect recurrence of disease, monitor the course of the disease and efficacy of the treatment in DTC. The average serum Tg test cost (without anti-Tg auoantibody test/information) in various Indian cities is approximately ₹1000. Therefore, to meet the growing demand of RMC-Tg KIT, automation in coating procedure would support large-scale production, thereby extending the benefit to a large number of patient population. The in-house developed kit is convenient, robust, cost-effective, fully automated for SR 300 RIA autoanalyzer and is being used satisfactorily in RMC for routine patient service for the last 8 years (Fig.8) by replacing

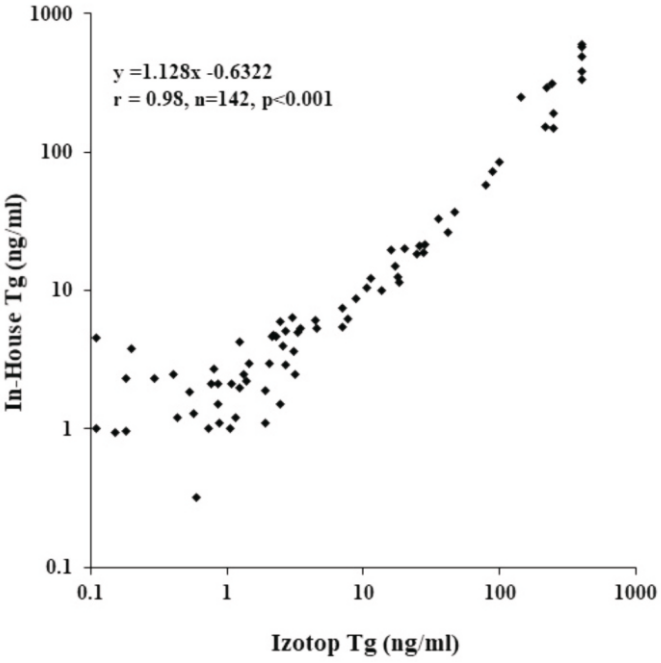

Fig.6: Scatter diagram for Tg by in-house RMC-Tg KIT and commercial TG IRMA kit (Izotop) in TgAb negative samples.

Fig.7: In-house made RMC-Tg KIT.

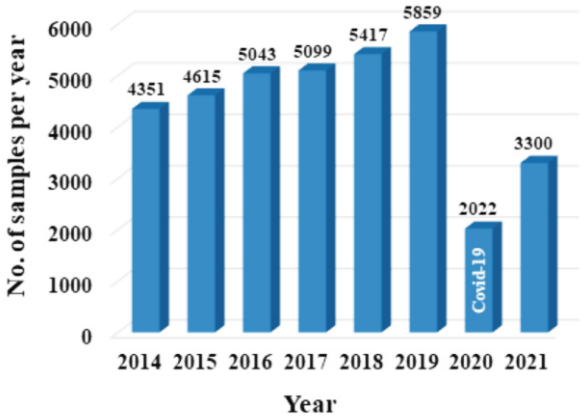

Fig.8: No. of serum samples sent for Tg analysis at RMC saw a drastic fall in 2020 and also in the following year, which is mainly due to the onset of Covid-19 pandemic.

the imported and expensive commercial Izotop TG IRMA kits. Indeed, Radiation Medicine Centre is the only Centre in India that produces and uses entirely indigenously developed RMC-Tg KITs for routine measurement of serum Tg in DTC patients (approx. 36,000 clinical samples analyzed so far). For the larger benefit of the society, we have thereby encompassed the application of radioisotope technology in the field of Nuclear Medicine.

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