Investigation of Methods to Remove Thyroglobulin Auto-antibodies Interference in Thyroglobulin Immunoassay.

Running title: Thyroglobulin antibodies interference in thyroglobulin assay

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Abstract

Background: Thyroglobulin (Tg) is a tumour marker used to monitor differentiated thyroid cancer (DTC). Antibodies to Tg (TgAb) may lead to underestimation of Tg using immunometric assays. Our aim was to investigate the elimination of TgAb interference in Tg assay via a) protein G magnetic beads (PGMB), and b) molecular weight (MW) filters.

Methods:
Samples with detectable TgAb and Tg (n=7) were extracted from Melbourne Health Pathology database. Aliquots were re-analysed as neat, post treatment with MW filtesr, PGMB or both using a chemiluminescent immunoassay.
Samples with detectable TgAb in patients with intact thyroid (n= 146) were selected to look for potential interference in Tg measurement. After de-identification, samples with low Tg (≤3 pmol/L) were treated with PGMB.

Results:
TgAb level was reduced by 46% post treatment with PGMB (p=0.034) and recovery of Tg was 70%. TgAb level was reduced by 41% post treatment with MW filter (p=0.016) and recovery of Tg was 50%. After MW filter and PGMB treatment, TgAb level was reduced by 72% (p= 0.028) but recovery of Tg was 23%.
Of the samples with elevated TgAb from patients with intact thyroid, 1 out of 21 samples with undetectable baseline Tg had a detectable Tg post PGMB treatment.

Conclusion:
PGMB or MW filters significantly reduced TgAb, but Tg recovery was poor using MW filters. Post PGMB treatment, 5% of samples with undetectable baseline Tg had a detectable Tg concentration. Further studies are required to assess the use of PGMB in patients with DTC and positive TgAb.

Introduction:

Serum thyroglobulin (Tg) is the only established tumour marker to monitor patients with differentiated thyroid cancer (DTC) [1,2]. Most laboratories measure Tg via immunometric assays (IMA) instead of radio-immunoassays due to the advantages of shorter incubation time and automation [3,4]. Antibodies to thyroglobulin (TgAb) has been shown to interfere with IMA methods, leading to underestimation of serum Tg [5] and potentially leading to relapsed cancers being missed [6]. This limitation significantly undermines the value of Tg in DTC management because 20-40% of patients have detectable TgAb [7].

The TgAb-Tg complex has been reported to prevent interaction with the reagent's capture and signal antibodies [5]. This interference is not related to TgAb concentration, and is determined by epitope characteristics with high avidity TgAb more likely to interfere with Tg IMA[8]. Recovery studies have been unreliable in predicting Tg interference in TgAb positive sera[3, 8,11,12]. The current paradigm is to perform TgAb with every Tg assay and to interpret the Tg result with caution when TgAb is detectable. However, there is poor concordance amongst commonly used TgAb assays in identifying TgAb positive sera [13]. The accurate measurement of Tg concentration is of paramount importance in management of patients with DTC and yet TgAb interference remains an unresolved issue. The American Thyroid Association Guidelines Taskforce in their 2009 statement has highlighted this as an area where work is urgently required [15].

Molecular weight (MW) filter is an established method to remove interfering antibodies in immunoassays, but its use in Tg measurement have not been reported [16]. The molecular weights of Tg and TgAb are 670 kD and 156kD respectively[21] and should be separable by a MW filter. Another method used to deplete serum IgG

class antibodies is protein G magnetic beads (PGMB) which binds the common region of IgG. A magnet is used to separate bound IgG from the serum. TgAb found in Hashimoto's sera is usually of the IgG class and has been associated with the different IgG subclasses to an extent which reflects the relative concentrations of the subclasses in serum (20). Protein G magnetic beads (PGMB) have strong affinity for the four human IgG subclasses. After incubation of PGMB and patient serum, a magnetic rack is used to separate the IgG bound to the PGMB and the IgG depleted sera. By depleting total IgG, TgAb will also be removed in the process and TgAb interference may be reduced. The aims of this study are 1) to explore the novel use of Protein G magnetic beads (PGMB) and MW filter to deplete TgAb, and 2) to assess the effect of TgAb depletion on samples with falsely low Tg results.

Materials and methods

To assess the optimal method to deplete TgAb concentration, 7 samples analysed at Royal Melbourne Hospital with a range of detectable TgAb (22.7-1175 IU/ml) and Tg levels (0.45- 15.3 pmol/L) were selected. All previously analysed samples were stored at -20◦C and re-analysed within a month. Re-analysed samples were thawed, centrifuged, and supernatant aliquotted into plain tubes. MW filters (Amicon Ultra 100KD MW filter, Millipore, MA, USA) were used according to manufacturer's instructions. PGMB (PureProteome protein G magnetic beads, Millipore, MA, USA) was resuspended according to manufacturer's instructions. Neat, MW filter treated samples, PGMB treated samples, and samples treated sequentially with MW filter and PGMB were analysed for Tg and TgAb using a chemiluminescent immunometric assay on the Siemens Immulite 2000 platform in a single run. The Tg assay was standardized against CRM 457 and interassay coefficient of variation (CV) was 4.8%

at 15 pmol/L, and 20% at 1.4pmol/L. Analytical sensitivity was 0.3 pmol/L. Normal reference range was up to 85 pmol/L for healthy subjects. The TgAb interassay CV at 43 IU/mL was 4.9%, analytical sensitivity was 2.2 IU/ml, normal reference range was < 55 IU/ml.

In order to identify samples with potential negative Tg interference, samples with positive TgAb (>20 IU/ml) and requests indicative of autoimmune thyroiditis were selected. Samples with Tg requested were excluded as they were likely from patients with differentiated thyroid cancer who already had thyroidectomies. Similarly, samples with clinical notes indicative of thyroxine replacement were also excluded as thyroidectomise might have taken place. Suitable samples (n= 146) were extracted from Royal Melbourne Hospital Pathology and Melbourne Pathology databases. Left-over sera were aliquotted to a plain tube and de-identified prior to analysis for baseline Tg and TgAb on the Siemens Immulite 2000 platform. Samples with positive TgAb (>20 IU/ml) and low Tg ≤3 pmol/L were then diluted 1 in 2 with phosphate buffer solution and treated with 100uL of suspended PGMB prior to separation. As these patients' requests suggested autoimmune thyroiditis as the indication for their blood tests, their thyroid should be intact and an undetectable or low Tg in the presence of TgAb suggests a possible negative TgAb interference.

This project was reviewed and approved by Royal Melbourne Hospital Ethics committee under the In Vitro Diagnostics Ethics code (QA2010095). It was supported by the Royal College of Pathologists of Australia Technical Assistance Grant. Neat and post treatment Tg and TgAb levels were analysed using the paired t-test, a value of $p < 0.05$ was considered significant. All statistical tests were performed using SPSS 16.1.

Results:

Seven samples with detectable TgAb and Tg were treated with MW filter, PGMB or both. The mean TgAb depletion using MW filter, PGMB and both methods were 41% ($p=0.02$), 46% ($p=0.03$) and 72% ($p=0.03$) respectively compared to untreated neat samples. The mean Tg recovery using MW filter, PGMB and both were 50% ($p<0.01$), 70% ($p<0.01$) and 23% ($p<0.01$) respectively compared to neat samples.

146 samples with clinical notes querying autoimmune thyroiditis with an average TgAb level of 350 IU/ml (SD= 638) were analysed for baseline Tg after de-identification. Fifty-two samples had a baseline Tg level of ≤3 pmol/L, of these, 21 samples had an undetectable Tg (<0.3pmol/L) with a mean TgAb level of 853 IU/ml (SD= 960).

Of the 31 samples with low but detectable Tg (0.3 -3 pmol/L), Tg recoveries post PGMB treatment were all less than 100% with a mean recovery of 26% ($p<0.01$) (Fig.3). Eighteen samples had a post treatment Tg of <0.3 pmol/L and a value of zero was assigned for calculation of the mean recovery. Tg recovery was not associated with TgAb depletion ($R^2= 0.01$).

Of the 21 samples with undetectable baseline Tg, 20 samples remained undetectable after PGMB treatment, one sample had a detectable Tg concentration of 0.66 pmol/L post treatment. The mean Tg Ab depletion of these samples post PGMB treatment was 52% ($p<0.001$). A limit of detection study was conducted on our Immulite 2000 analyser by performing Tg 31 times on the zero calibrator. The analytical sensitivity was 0.25pmol/L (mean=0.11, SD=0.07). A value of 0.66 pmol/L can be distinguishable from zero with a confidence limit of 97%.

Discussion:

While novel tumour markers such as TSH receptor mRNA is under development, serum Tg remains the only established tumour marker in the follow-up of DTC patients. The reliability of Tg IMA measurements is hampered by the presence of TgAb in serum (18), this interference remains unresolved despite use of high salt buffer (22) to dissociate the Tg-TgAb complex and the epitope selection approach in reagent manufacture. The quantification of Tg by peptide immunoaffinity enrichment liquid chromatography tandem mass spectrometry is not affected by TgAb, but was limited by its poor analytical sensitivity (19). Radioimmunoassays are more robust to TgAb interference, however they are no longer commonly available due to lack of automation.

Optimal removal of TgAb interference requires a method which depletes TgAb 100% while achieving 100% recovery of Tg. This is impractical as any treatment will cause some loss of Tg recovery. We found that the maximum TgAb depletion was achieved using a combination of MW filter and PGMB, but Tg recovery was poor therefore negating this method as a practical solution to monitor patients with resected DTC whose unstimulated Tg is at low concentration. PGMB treatment was superior to MW filters in terms of Tg recovery (70% vs 50%), while achieving a similar degree of TgAb depletion (46% vs 41%).

As there is conflicting data on the reliability of recovery studies in the assessment of TgAb interference, we assessed TgAb positive sera from patients with autoimmune thyroiditis whose requests and test patterns suggested the presence of an intact thyroid. Of the 146 samples extracted, 21% had a low Tg (< 3 pmol/L) and 14 % had an undetectable Tg inconsistent with the presence of an intact thyroid. PGMB treatment of samples with a low but detectable Tg resulted in a mean Tg recovery of 26%. Tg recovery was not related to the degree of TgAb depletion in these samples, therefore Tg

was not lost via the depletion of the TgAb-Tg complex due to PGMB separation. 5% of the samples with undetectable baseline Tg had a detectable Tg level post PGMB treatment. The post treatment TG value of 0.66 pmol/L was distinguishable from zero with a confidence interval of 97% using data from our in-house analytical sensitivity study.

Our study is limited by its small number and only one sample had a detectable Tg post PGMB treatment. As the prevalence of falsely low Tg in patients with autoimmune thyroiditis is unknown, a power analysis cannot be performed beforehand. Our findings will also need to be replicated in DTC patients with documented falsely low Tg levels as their TgAb epitope repertoire might be different to that of autoimmune thyroiditis patients. Similarly, serum Tg isoforms in autoimmune thyroiditis might differ from those found in thyroid cancer [22], resulting in differential recovery using PGMB. The assumption of intact thyroid in the thyroiditis cohort was made from clinical notes on the request forms and test patterns. Some of these patients might have had a thyroidectomy and the low Tg concentrations might have been true results. However, it is unusual to request TgAb and TPO Ab in patients post thyroidectomies without a Tg request.

In conclusion, we have shown that a novel method to deplete TgAb via protein G magnetic beads has good Tg recovery and might be useful in detecting negative interference in Tg immunometric assays. Larger prospective studies are required to assess the use of PGMB in patients with DTC and positive TgAb.

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