interval of 3.97–7.79 mmol/L, using the nonparametric procedure. The other three procedures provided similar, although slightly narrower, intervals. (All of the tables are available by request. Each table consists of 27 analytes, 5 of which are hematological.)

Where a transformation of the data for a particular test to a gaussian distribution was not possible or if the Anderson-Darling test rejected normality, the reference interval was not computed and the appropriate cell was left blank. It is noteworthy that in approximately one-third of the cases, no suitable transformation was found to allow for use of traditional normal theory methods. However, it is reassuring that when a successful transition was achieved, the resulting reference interval was comparable to that of the transformed robust (which requires only symmetry) as well as the untransformed robust method. It is also noteworthy that the robust method, although designed primarily for right-skewed populations, gives results that are comparable to the other, traditional methods for left-skewed data such as hemoglobin.

The reference interval width for the W group was compared to that of the T group by using their ratio because this dimensionless quantity allows for comparisons across analytes. For the 27 analytes tested, the interval width for the W data was on average 25% wider than the interval width based on the T data using either the nonparametric or robust analysis. To evaluate the effect of using different methods of estimating the reference interval, the ratio of the interval width calculated by the nonparametric procedure to that of the transformed robust (which requires only symmetry) as well as the untransformed robust method. It is also noteworthy that the robust method, although designed primarily for right-skewed populations, gives results that are comparable to the other, traditional methods for left-skewed data such as hemoglobin.

Table 1. 95% reference intervals for males 50–59 years of age.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>T group (n = 210)</th>
<th>W group (n = 178)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nonparametric</td>
<td>Robust</td>
</tr>
<tr>
<td>ALT, a U/L</td>
<td>9.65</td>
<td>9.65</td>
</tr>
<tr>
<td></td>
<td>55.21</td>
<td>53.36</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>3.97</td>
<td>3.97</td>
</tr>
<tr>
<td></td>
<td>7.79</td>
<td>7.72</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>4.64</td>
<td>4.64</td>
</tr>
<tr>
<td></td>
<td>13.73</td>
<td>11.53</td>
</tr>
<tr>
<td>Hemoglobin, mmol/L</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>2.61</td>
<td>2.57</td>
</tr>
</tbody>
</table>

*ALT, alanine aminotransferase.

Our recommendation for the development of reference intervals includes the use of both nonparametric and robust estimators where the relationship between the intervals estimated by the two methods can be used as an “ad hoc” estimate of the reference population quality. Evaluation of the reference intervals by both of these techniques should yield similar results. If they do not agree, then consideration must be given to the possibility that a significant part of the sample could have diseased individuals or that multiple populations are being measured.

References

Measurement of Thyroid-stimulating Hormone Receptor Autoantibodies by ELISA, Jane Bolton, Jane Sanders, Yasuo Oda, Ceri Chapman, Reiko Konno, Jadwiga Furnaniak, and Bernard Rees Smith (R SR Ltd., Avenue Park, Pantywn, Cardiff CF23 8HE, UK; 2 Cosmic Corporation, Tomisaka Bldg., 2-7-3 Koishikawa, Bunkyo-ku, Tokyo 112, Japan; * author for correspondence: fax 44-1222-732704)

Hyperthyroidism in Graves disease is attributable to autoantibodies to the thyroid-stimulating hormone receptor (TSHR), and measurement of these TSHR autoantibodies (TRAbs) can be useful in disease diagnosis and
management (1–4). Usually TRAbs are detected by bioassays based on cultured cells or by receptor assays based on 125I-labeled TSH (1–4). The most widely used receptor assay (5) uses detergent-solubilized porcine TSHR with TRAbs to inhibit the TSHR-125I-TSH interaction, and assay excess TSH-bi was removed (100 water. The peroxidase substrate tetramethylbenzidine incubation was continued for 20 min. The wells were then absorbance of; absorbance at 450 nm.

The C-terminal end of the porcine TSHR (last 160 amino acids) (3) was expressed in Escherichia coli as a fusion protein with glutathione S-transferase and used to produce MAb as described previously (7). One of the MAb had a relatively high affinity (5 × 10 9 L/mol) for deter- gent-solubilized porcine TSHR, and the purified IgG was used to coat ELISA plate wells by incubation overnight at 4 °C. In these experiments, 100 μL of 0.01 g/L antibody in 100 mmol/L NaHCO 3 , pH 9.2, was used. After washing and coating with 10 g/L bovine serum albumin (Sigma- Aldrich), the plates were washed again with assay buffer (10 mmol/L Tris-HCl, pH 7.4, 50 mmol/L NaCl, 1 g/L bovine serum albumin, 1 mL/L Triton X-100). Detergent-solubilized porcine TSHR (100 μL) diluted in assay buffer was then added, and the plates were incubated for 30 min with shaking at room temperature. The TSHR preparation was then removed, and the wells were washed with assay buffer. The receptor-coated wells were then used imme- diately or dried, sealed in foil pouches, and stored at 2–8 °C.

In the assay, 100 μL of test sera and 10 μL of mouse serum (to neutralize any anti-mouse IgG present in the test sera) were added to duplicate receptor-coated wells, followed by incubation at room temperature for 2 h. Sera were then removed (without washing at this stage), and 100 μL (5 ng) of biotinylated bovine TSH (TSH-bi; RSR Ltd.) in assay buffer added. After further incubation for 15 min at room temperature, excess TSH-bi was removed (without washing at this stage), 100 μL of streptavidin-peroxidase conjugate (Sigma-Aldrich) was added, and incubation was continued for 20 min. The wells were then washed once with assay buffer and once with distilled water. The peroxidase substrate tetramethylbenzidine (100 μL) was added to the wells, followed by, after 20 min, the addition of 2 mol/L H 2 SO 4 and measurement of absorbance at 450 nm.

In the presence of sera from healthy blood donors, an absorbance of ~2 was observed. This was reduced in a dose-dependent manner to ~0.3 by TRAbs or by bovine TSH. For example, thyroid-stimulating antibody first international standard 90/672 (National Institute for Bio- logic Standards and Controls, Hertfordshire, UK) at 3, 10, and 30 units/L gave 15%, 37%, and 70% inhibition of TSH binding, respectively [inhibition of TSH binding expressed as: 100 × (1 − ratio of absorbance at 450 nm of test sample to absorbance at 450 nm of a pool of healthy blood donor sera)].

Sera from individual healthy blood donors (n = 65), double-stranded DNA antibody-positive patients with systemic lupus erythematosus (n = 10), rheumatoid fac- tor-positive patients with rheumatoid arthritis (n = 10), and patients with Hashimoto thyroiditis (n = 15) showed inhibition of TSH binding of <10%.

We compared the ELISA and the conventional TRAb receptor assay based on 125I-labeled TSH and PEG precip- itation (5) in 56 sera from patients suspected of having Graves disease (Fig. 1). Of the 56 sera studied, 38 were clearly positive by 125I assay, and the same 38 were clearly positive by ELISA (inhibition of TSH binding >15% in both assays). Two sera were borderline positive in both assays (12–13% inhibition), and one serum was borderline positive by 125I assay (11% inhibition) but negative by ELISA (3% inhibition). At this stage it was not clear which result (borderline positive or negative) would better reflect the outcome of treatment. The remaining 15 sera were negative in both the radioactive assay (inhibition of binding, −4% to 8%) and the ELISA (inhibition of binding, −6% to 9%). We used the same 56 sera to compare the ELISA and a TRAb bioassay based on stimulation of cAMP production in isolated porcine thyroid cells (8) (reagents from Yamasa Ltd.); r = 0.73. Thirty-four of the 56 sera were positive by bioassay compared with 38 of 56 by ELISA. One sample was positive by bioassay (285% stimulation; positive = greater than 180%) but only border- line positive by ELISA (12% inhibition) and by radio- active assay (12% inhibition). The 56 patients in the study were receiving or had received treatment for hyperthy- roidism; consequently, in the case of the five sera with discrepant bioassay and ELISA results, it was not clear which result better reflected the underlying disease activity [see Refs. (1, 2) for a review of the relative clinical

![Fig. 1. Comparison of TRAbs by ELISA and radioactive assay in 56 sera from patients suspected of having Graves disease.](image-url)
effectiveness of TRab measurement by inhibition of TSH-binding assay and by bioassay].

In terms of TRab ELISA precision, typical variations in absorbances at 450 nm (in the same assay run) were 1.67 ± 0.05, 1.1 ± 0.04, 0.76 ± 0.025, and 0.51 ± 0.028 (means ± SD; n = 28).

Intraassay imprecisions (CVs) were 9.0%, 4.5%, and 2.3% at mean inhibitions of TSH binding of 30%, 42%, and 71%, respectively (n = 28), and interassay CVs were 6.8%, 3.3%, and 2.3% at mean inhibitions of TSH binding of 24% (n = 10), 45% (n = 6), and 69% (n = 10), respectively. These values of intra- and interassay imprecision are similar to those observed for the radioactive assay (5).

Occasional sera from patients with Graves disease contain TSH antibodies (9, 10) that form complexes with labeled TSH in the PEG-based receptor assay for TRAb. These TSH-antibody complexes are precipitated with PEG in addition to TSHR-TSH complexes, giving an increase in the ELISA as compared with healthy blood donor sera. Consequently, TRAb measurements with the PEG method are difficult to make in samples containing TSH antibodies. To investigate the effects of TSH antibodies in the ELISA, measurements were made in sera from 48 treated Graves disease patients selected for the presence of antibodies reactive with labeled bovine TSH. The effects of anti-TSH antibodies clearly evident in the PEG method (mean inhibition of TSH binding, −33.3%; range, −13% to −87%) were not observed in the ELISA (mean inhibition of TSH binding, 18.7%; range, −0.4% to 47%). This was presumably attributable to the removal of the test serum samples containing TSH antibodies after incubation in the receptor-coated wells. Consequently, TRAb concentrations were measurable in samples containing TSH antibodies with the ELISA, and in the series studied, 38 of 48 sera showed ≥10% inhibition (Table 1).

Our results indicate that porcine TSHR immobilized on ELISA plates, using a MAb to the receptor’s COOH terminus, and TSH-biotin can be used to create a TRAb ELISA. The ELISA has similar sensitivity and precision to the current radioactive test but has some major advantages, including easy automation and absence of interference from antibodies to TSH.

Table 1. Inhibition of TSH binding by sera (from 48 patients with Graves disease) containing TSH antibodies.

<table>
<thead>
<tr>
<th>Inhibition of TSH binding (range), %</th>
<th>No. of patients in range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PEG method</td>
</tr>
<tr>
<td>&gt;15</td>
<td>0/48</td>
</tr>
<tr>
<td>10–14</td>
<td>0/48</td>
</tr>
<tr>
<td>&lt;10</td>
<td>48/48</td>
</tr>
</tbody>
</table>

References


Evaluation of an Automated Enzyme Inhibition Assay for the Detection of Anti-Mitochondrial M2 Autoantibodies, Patrick Schmit, Georges Gilson, and René L. Humbel

Primary biliary cirrhosis (PBC) is a chronic disease characterized by portal inflammation and necrosis of small intrahepatic bile ductules (1). PBC is an irreversible condition, and destruction of the bile ductules leads to progressive cholestasis and fibrosis, and may eventually lead to the development of cirrhosis. PBC is most likely an autoimmune disorder (2), and anti-mitochondrial antibody (anti-M2) has been a diagnostically very useful marker: several studies have reported a positivity rate for anti-M2 of >95% in biopsy-confirmed PBC patients (3). The major mitochondrial antigen was identified as the 74-kDa E2 subunit of the pyruvate dehydrogenase complex (PDC), a member of the 2-oxoacid dehydrogenase complex (PDC), a member of the 2-oxoacid dehydrogenase complex family (4). The traditional technique for the detection of anti-M2 is immunofluorescence (5), but recently new serological assays, such as ELISA, immunoblotting, and enzyme inhibition (EI), have been developed (6, 7). A miniaturized EI assay for performance on microtiter plates has already been described (8, 9). In this study we evaluated a new commercially available and completely automated EI assay manufactured by Trace Scientific (Victoria, Australia) and compared it to the immunofluorescence and ELISA techniques performed in our laboratory.

The TRACE enzymatic procedure is a unique method based on the PDC inhibitory properties of the principal