C-67: *Direct epitope recognition assay for TSH receptor autoantibodies causing Graves' disease demonstrates higher diagnostic accuracy than indirect assays based on TSH displacement*

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Objective:

In Graves' disease (GD) hyperthyroidism is caused by stimulating TSH receptor (TSHR) autoantibodies (sTRAb). TSHR is a seven transmembrane receptor with a large extracellular domain presenting epitopes for stimulating as well as blocking TSHR autoantibodies (Fig. 1, left). Current TSH displacement assays (TDA) quantify indirectly all TSHR autoantibodies (Ab) including blocking Ab. Our novel direct epitope recognition assay (DERA) was developed for direct detection of sTRAb (Fig. 1, right).

Relevance:

GD is a common cause for hyperthyroidism. In routine practice values determined by TDA conflict in several cases with clinical diagnosis.

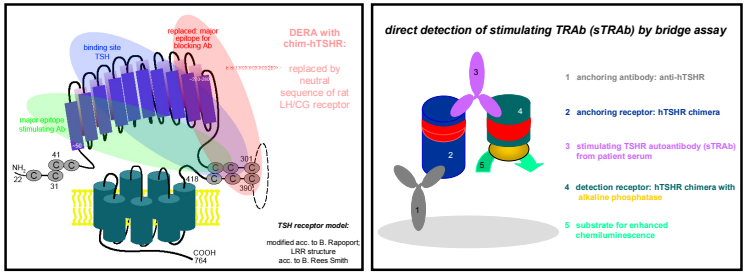
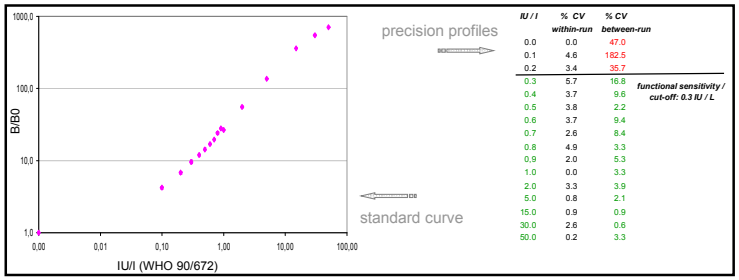


Figure 1: *LEFT*: TSH receptor model illustrates major epitopes for stimulating and blocking TSHR autoantibodies. In chim-hTSHR the latter one is replaced by the neutral sequence of the rat LH/CG (luteinizing hormone/choriogonadotropin) receptor. The TSH binding site is indicated as well. *RIGHT*: Direct detection of TSHR stimulating autoantibodies (sTRAb) causing GD using bridge assay technology with chim-hTSHR constructs (sTRAb-DERA). RLU = relative light units

Methodology:

DERA is performed by bridge technology on microplates using recombinant chimeric hTSHR-rLH/CG receptors (chim-hTSHR) where the major epitope for blocking Ab is replaced by the corresponding neutral sequence of the LH/CG receptor. An antibody anchors chim-hTSHR to microplates. One arm of the sTRAb binds to chim-hTSHR, the second arm bridges to a chim-hTSHR fused with alkaline phosphatase. Applying chemiluminescent substrate sTRAb were quantified using a plate luminometer (Fig. 1, right). DERA is performed at ambient temperature with incubation of the second chim-hTSHR at 37°C. Chim-hTSHR showed prolonged thermostability beyond assay duration.



IU / l	% CV within-run	% CV between-run
0.0	0.0	47.0
0.1	4.6	182.5
0.2	3.4	35.7
0.3	5.7	16.8
0.4	3.7	9.6
0.5	3.8	2.2
0.6	3.7	9.4
0.7	2.6	8.4
0.8	4.9	3.3
0.9	2.0	5.3
1.0	0.0	3.3
2.0	3.3	3.9
5.0	0.8	2.1
15.0	0.9	0.9
30.0	2.6	0.6
50.0	0.2	3.3

Figure 2: DERA standard curve ranging from 0.3 IU/l to 50.0 IU/l using WHO standard 90/672 for thyroid stimulating autoantibodies and precision profiles for within-run as well as between-run based on five independent runs with triplicate samples.
B/B0: RLU mean of each standard value divided by RLU mean of zero standard (n = 3).
Functional sensitivity of DERA: 0.3 IU/L.

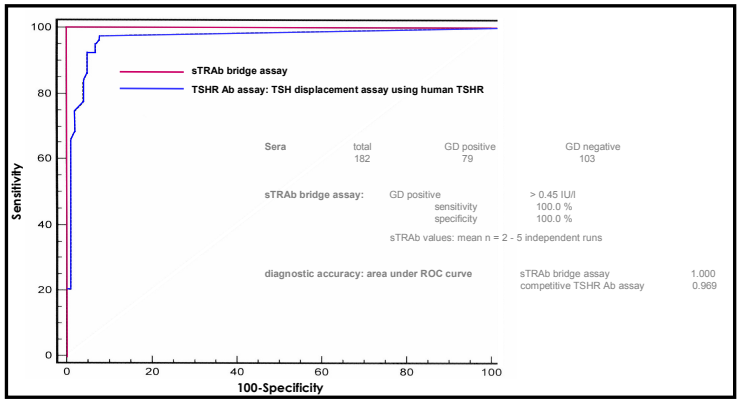


Figure 3: Comparative ROC plot analysis of GD patient and normal sera: the sTRAb bridge assay shows higher diagnostic accuracy (1.000) compared to a commercial TSHR Ab assay based on TSH displacement at the human TSHR (0.969). In the sTRAb bridge assay criterion (treshold concentration) for correctly classifying subjects into positive and negative group was determined as sTRAb >0.45 IU/l with 100.0 % diagnostic sensitivity & 100.0 % specificity. Assuming 10 % imprecision the grey zone is only 0.4 – 0.5 IU/l.

serum	autoimmune process #	sTRAb positive >0.45 IU/l	TRAb * 1.0 < grey zone ≤ 1.5 IU/l	improvement by sTRAb
1	+	2.35	1.10	explicit diagnosis
2	-	*0.0*	1.40	
3	-	0.45	1.20	
4	-	0.21	1.40	
5	+	2.26	1.50	
6	+	1.14	1.30	
7	+	1.93	1.20	
8	+	0.93	1.30	
9**	-	*0.0*	18.80	no false positive based on serum Ab concentration: measured TRAb do not induce hyperthyroidism; e.g. blocking Ab
10	-	0.37	2.20	
11	-	0.12	2.20	
12	-	0.43	2.80	
13	+	1.10	<1.0	no false negative based on serum Ab concentration
14	+	1.54	<1.0	
15	+	5.50	1.60	well-defined diagnosis
16	-	*0.0*	1.80	no interference by Thyroiditis de Quervain

* : TRAb by commercial TSH displacement assay using human TSHR and labelled bovine TSH
\# : activity evaluated by clinical diagnosis (tachycardia, ATD treatment) and laboratory tests (TSH, T3, T4): – = negative, + = positive
0.0 : sTRAb value < negative standard
** : thyroxin substitution essential for normal thyroid metabolic status of the patient

Table 1: Portrayal of patient sera with discrepancies in diagnosis based on conventional TRAb measurement compared to clinical diagnosis of the autoimmune process. Determination of sTRAb activity by sTRAb bridge assay improves diagnosis for GD based on serum parameter reflecting higher diagnostic accuracy of the sTRAb bridge assay (DERA).

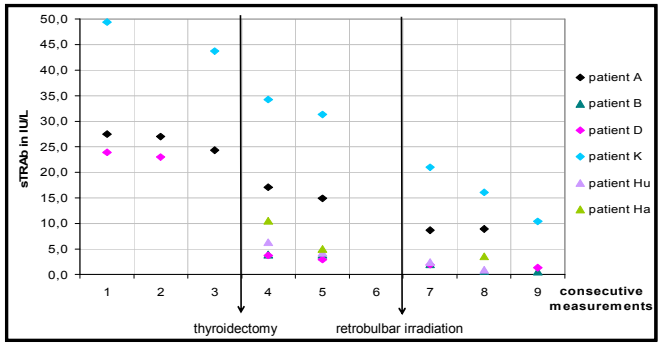


Figure 4: Patients with active thyroid associated ophthalmopathy (TAO) were treated by thyroidectomy and retrobulbar irradiation. Both treatments affect the autoimmune process as indicated by decreasing sTRAb values supporting hypothesis of active autoimmune process in orbital tissue.

Validation:

The range of detection was established using WHO standard 90/672 for thyroid stimulating autoantibodies showing between-run precision (different lots and days included) of CV <20 % from 0.3 – 50.0 IU/l at within-run CV <10 % (Fig. 2). The power of DERA to distinguish between sera positive or negative for sTRAb related to clinical diagnosis was tested via ROC plot analysis using 182 sera (79 patient, 103 sera GD negative). Autoimmune activity was indirectly quantified by clinical diagnosis (e.g. tachycardia), laboratory parameters (TSH, T3, T4), and drug treatment. With a decision limit of >0.45 IU/l DERA showed 100.0 % diagnostic sensitivity and 100.0 % specificity in ROC plot (Fig. 3). Compared to TDA, DERA demonstrated a higher diagnostic accuracy expressed as area under ROC curve: 1.000 vs. 0.969 (Fig. 3). Considering a between-run imprecision of 10 % CV at the decision limit (see precision profile Fig. 2) the DERA features a rather small grey zone of 0.4 - 0.5 IU/l compared to that broader one of the TDA comprising 1.0 - 1.5 IU/l. Comparison of both methods showed a good correlation of values (r=0.82). Forming three goups, namely 1st sTRAb higher (20 sera), 2nd sTRAb lower (36 sera), 3rd sTRAb equal to TDA values (126 sera), the correlations significantly increased (r=0.98/r=0.92/r=0.99). For 5 patients with negative clinical diagnosis (1 with Thyroiditis de Quervain) the TDA result was positive whereas DERA value was correctly negative. One neg. patient treated with thyroxine for hypothyroidism to reach euthyroidism gave proof that his TDA value (18.8 IU/L) was caused by blocking TSHR Ab. For 2 patients with positive DERA and negative TDA values the existence of sTRAb is confirmed by positive clinics. Further improvement by DERA is the diagnostic differentiation within TDA grey zone (1.0 - 1.5 IU/L) for 8 sera. In 4 patients with active thyroid associated ophthalmopathy sTRAb levels decreased after thyreodectomy. After retrobulbar irradiation a further significant decrease was detected (Fig. 4).

Conclusion:

DERA (direct epitope recognition assay) is a new high sensitive and specific *in vitro* assay suitable for routine clinical diagnostic in Graves' disease. Measuring directly only GD causing TSHR autoantibodies (sTRAb) its diagnostic accuracy exceeds that of current TSH displacement assays with indirect quantification. Differences between direct and indirect assay values are suggested to be due to I) the measurement of blocking and stimulating antibodies as well as to II) only partial sharing of epitopes between labeled TSH and TSHR antibodies in the TSH displacement assay. The sTRAb assay is suitable for fully automatisation.
Substantial decrease of sTRAb values after retrobulbar irradiation support hypothesis of an active autoimmune process in orbital tissues accessorily to the thyroid as an autoantigen.