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Comperative study of biotin, streptovidin -ELISA and peroxidase - ELISA for the estimation of TSH in thyroid diseases

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Abstract: ***Background:*** *Current medical needs dictate that laboratories offer thyrotropin (TSH) assays that can reliably measure low TSH. The protocol for determining the functional sensitivity of TSH assays specifies analyses of serum samples with two reagent lots over a 6- to 8-week period.*
Methods: *Accordingly, the objective of this study was to evaluate the efficacy of two TSH ELISA assay methods. Sensitivity was evaluated by comparing the level of TSH hormone by testing a range of human serum samples with at least 2 reagent lots for each method.*
Results: *Both ELISA methods were able to detect hormone levels in the sera, but detection was very significantly (t test, P<0.05) greater with the biotin- streptavidin ELISA in sera from human serum samples than in the peroxidase ELISA. The sensitivity of the biotin- streptavidin ELISA was further enhanced by redefining the calibration curve pattern characteristics, using low end calibrators. With the usage of several statistical considerations (regression analysis) and mathematical curve fitting models lower levels of TSH hormone could be successfully evaluated for early hyperthyroid case detection.*
Conclusion: *Second generation TSH assays can be modified for lower detection sensitivity by redefining calibration curve pattern characteristics, using low end calibrators.*
Key Words*: ELISA, Calibration curve, regression model,hyperthyroidism*

I. Introduction

Over the past five decades, improvements in the sensitivity and specificity of thyroid test methodologies have dramatically impacted the clinical strategies for detecting and treating thyroid disorders.

Currently, thyroid testing is performed on serum specimens using either manual or automated methods employing specific antibodies. Methodology is still evolving as performance standards are established by the professional organizations and new technology and instruments are developed by manufacturers. The enhanced sensitivity and specificity of TSH assays have greatly improved the assessment of thyroid function tests.

The first-generation radioimmunoassays, launched in the late 1960s, had a functional sensitivity limit of 1 to 2 mU/L. The current widely used immunometric or sandwich assays (second generation) have a functional sensitivity of 0.1 mU/L. Further developments have led to third-generation chemiluminescence assays, with about a ten-fold improvement in functional sensitivity (1). Extremely sensitive TSH assays are now available; the 4th/5th generation assays can detect TSH levels as low as 0≤0.004mU/L. However, for practical purposes, TSH values of ≤ 0.1mU/L are considered sufficient (2-7).

The current TSH detection kits approved under government set up are the second generation sandwich assays. The lower limit of detection of these kits as claimed by most of the manufacturers is 0.027 µlU / ml TSH.

It is recommended that the functional sensitivity of the TSH assay be verified independently of the manufacturer's claim by use of human serum pools. At least 10 different runs spaced over 6-8 weeks with at least two different lots of reagents and two different instrument calibrations during the test period should be performed (8). We therefore conducted experiments according to these guidelines, using 2 TSH methods.

We also compared the mean TSH results, using the same serum pools that were used to assess imprecision, to evaluate the agreement of these methods for low TSH concentrations.

However, after rigorously following the protocol guidelines almost all the assays many a times failed to detect very low end samples and many a times no numerical value could also be assigned to such patient sample. Thus, a need to modify the present assays with higher low end detection limits was essential. Accordingly, two ELISA based TSH diagnostic kits were evaluated.

Enzyme-linked immunosorbent assays (ELISA) are widely used to detect cytokines, clinical diagnostic markers, food allergens, and hormones in biological samples. ELISA is a highly specific and sensitive method that uses an enzyme linked antibody to detect and quantify these antigens.

Sandwich ELISA is the method of choice for TSH estimation in routine laboratory immunodiagnostics.

Among the Present day second generation assays numerous strategies have been devised to increase the sensitivity .One approach to increasing the sensitivity of EIAs is simply to increase the incubation period of the reporter enzyme with its substrate, provided that appropriately stabilized reagents are available. For example, a typical protocol involving incubating alkaline phophatase (ALP) with p-nitrophenyl phosphate for 60 min can result in immunoassay sensitivity for thyrotropin (TSH) of better than 0.1 milli-int. unit/L. Another general approach is to increase the number of reporter molecules per analyte molecule. Examples include polymerized enzymes**,** enzyme-anti-enzyme complexes**,** liposome-entrapped enzymes , and biotin-avidin amplification.. However, many of these assay strategies have higher nonspecific binding, with only modestly increased signal enhancement (10-13).

Accordingly, an attempt was made to decipher the comparative efficacy of two TSH kits one employing sandwich peroxidase enzyme linked immunosorbant method and the other employing sandwich biotin-streptavidin enzyme linked immunosorbant method for detecting TSH.

Calibration curve of both the methods was analyzed statistically using linear, non linear regression analysis and 4 parameter logistic log regression fit. Further, the necessity to redefine the calibration curve pattern for hyperthyroid case detection was also done by introducing low end calibrators; this significantly improved the overall sensitivity for TSH analysis in sera samples.

II. Materials and Methods

The following methods were evaluated TSH diagnostic kits employing peroxidase-ELISA detection and biotin-streptavidin ELISA detection system. Instruments used were Erba LisaScan II (Erba diagnostics) and Lablife elite 96s.The lower detection limit of both the assays were 0.027µLU / ml according to manufacturers product insert. Two different lots of reagents were used for each method. According to the manufacturers' information, all assay calibrators are traceable to the WHO Second International Reference Preparation 80/558.

Assay imprecision was assessed by use of commercial quality-control materials, Lyphocheck Levels 1-3 (Bio-Rad Laboratories). Each control material was analyzed by each method in duplicate per run. Daily runs were performed 2 days a week over a period of 3 weeks for each lot of reagent for a total of 24 replicates for each control.

Calibration profiles of TSH using both the methods were plotted following the product insert instruction of the respective kits (14, 15).

However, there were occasional cases when the TSH levels were so low that a numerical value could not be assigned to the sample. Also assigning correct value to borderline cases was becoming a matter of concern. Since, the need for correct value assignment will have a direct impact on clinical case detection a need to further modify the low end calibration curve profile was undertaken. Further, calibration curve included the insertion of 0.17, 0.25, 0.50, 1.25, 2.5, 5 and 10µU/ml calibrators. 0.17, 0.25 calibrators were prepared by serial dilution of 0.5 calibrator as provided by the kits respectively, similarly 1.25 calibrator was prepared by half dilution of 2.5 calibrator as provided by the kits respectively.

Statistical Analysis: All the statistical analysis including correlation and regression and curve fitting analysis was performed using the Graphpad prism version 5 software.

III. Results and Discussion

Temperature plays a pivotal role in enzyme substrate reaction in ELISA methodology. Accordingly, the optimum temperature of 22^{o}C was selected as per the product insert of both the methods.

The Performance of both the ELISA was calibrated using 0-40 µUL/ml TSH standards. The calibration curve was fitted using 4 parameter logistic model analysis is tabulated in Table1.

	SS	SYX	R2
Biotin Streptavidin	0.0001326	0.005758	0.9998
Peroxidase ELISA	0.0002246	0.007493	0.9997

Table 1: Evaluation of goodness of fit of the calibration curve using the 4 parameter logistic model analysis.

A comparison of goodness of fit of both the calibration curve generated by the two methods respectively revealed the sum of square of the distances of the points from the curve of Biotin Streptavidin ELISA to be less than Peroxidase ELISA. Further, The value sy.x is the standard deviation of the residuals of Biotin Streptavidin ELISA is also less than the latter. Further, the R2 of the former was better than the latter. These results confirm the superior calibration curve fit of the former.

The correlation analysis of O.D vs. Conc. of the two methods also revealed a better value of Correlation Coefficient being $r^2 = 0.99$ for the Biotin Streptavidin ELISA as compared to $r^2 = 0.95$ for Peroxidase ELISA upon 1 hr incubation.

However, with 1 hr incubation most of the low end samples could not be assigned numerical values therefore, the need to optimize assay conditions was required. As recommended by the product insert of both the

methods detection of lower TSH levels could be evaluated with longer incubation period. Accordingly, the Performance of both the ELISA was calibrated using 0-40 µUL/ml TSH standards with 2 hrs incubation. The calibration curve was fitted using 4 parameter logistic model as depicted in Fig 1. Correlation Coefficient of the Biotin Streptavidin ELISA was 0.99 as compared to 0.98 of Peroxidase ELISA upon 2 hr incubation as depicted in Table 2.

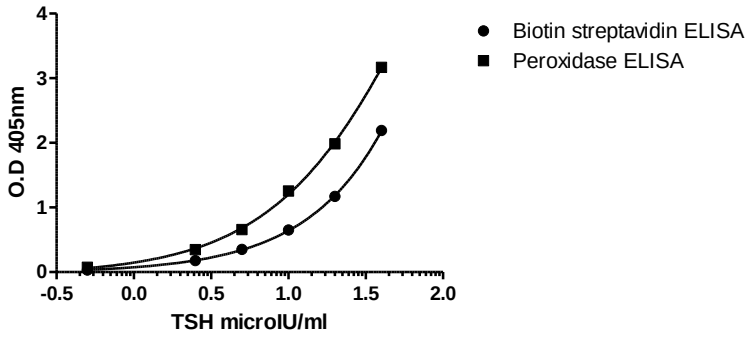

Fig 1: Comparison of Biotin Streptavidin vs Peroxidase ELISA with 2 hr incubation period using 4 parameter logistic model

	Biotin Streptavidin	**Peroxidase ELISA**
Pearson r	0.9986	0.9862
95% confidence interval	0.9898 to 0.9998	0.9058 to 0.9980
P value (two-tailed)	< 0.0001	< 0.0001
Is the correlation significant? (alpha=0.05	Yes	Yes
R square	0.9971	0.9725

Table 2: Correlation analysis of the Biotin Streptavidin vs Peroxidase ELISA with 2 hr incubation The performance of both the ELISA methods was evaluated by calculating the CV as tabulated below.

TSH mean µLU/ml	**Biotin Streptavidin** CV%	**Peroxidase ELISA** CV%
0.5	1.88	11
2.5	3.09	12
5	1.38	6.6
10	3.75	6.9
20	1.34	9.3
40	0.17	11.2

Table 3: Performance characteristics of the two ELISA methods and their CV

The *coefficient of variation* (CV), also known as "relative variability", equals the standard deviation divided by the mean. The former method has a relatively lower CV across the entire working range of TSH confirming good assay reproducibility.

To achieve an analytical method comparison, The Bland-Altman difference plot was performed between the two ELISA methods.

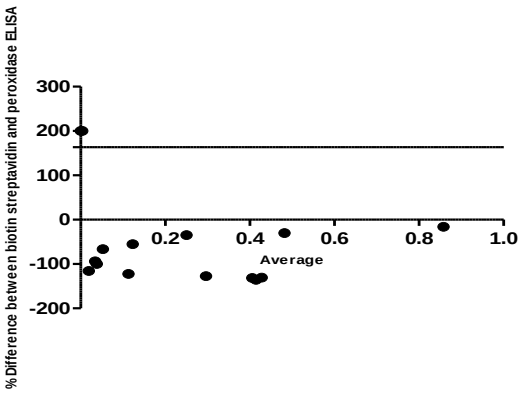

Fig 2: Bland-Altman plot displaying the per cent difference values between the two methods (y axis) and their average (x axis).

Bland Altman analysis revealed for low TSH values the difference was too high between the methods whereas the difference was not so relevant for high TSH values confirming the precision of the former method to the latter at lower working range.

A few Patient samples were run and the unknown concentrations were interpolated. Both the calibration curves of the two methods were subjected to non linear regression analysis using the cubic spline function and the results obtained are tabulated below.

Patient Sample	Streptavidin	Interpolated concentration	Peroxidase	Interpolated concentration
Unknown sample 1	0.1390	0.955856	0.198	0.6725614
Unknown sample 2	0.1330	0.9135185	0.204	0.6960741
Unknown sample 3	0.1080	0.7377173	0.149	0.4849988
Unknown sample 4	0.0190	0.1178439	0.031	0.05694508
Unknown sample 5	0.0440	0.2913026	0.066	0.1819033
Unknown sample 6	0.0070	0.03465668	0.013	n.v
Unknown sample 7	0.1480	1.019482	0.207	0.7078649

Table 4: Comparative Interpolation of results of patient samples using the cubic spline model

As observed from the above data there is a significant ($P<0.05$) signal enhancement of low end values with the streptavidin ELISA as compared to peroxidase ELISA. Sample no 6 could not be assigned any numerical value as per the peroxidase method, however, a low value was obtained with the other method, confirming the superiority of the former.

In an effort to further improve the low end data determination more calibrators were included accordingly performance of both the ELISA was calibrated using 0, 0.17, 0.25, 0.50, 1.25, 2.5, 5 and 10µU/ml TSH calibrators. Correlation Coefficient of the Biotin Streptavidin ELISA was 0.999 as compared to 0.995 of Peroxidase ELISA. The calibration curve was fitted using 4 parameter logistic model Fig 3.

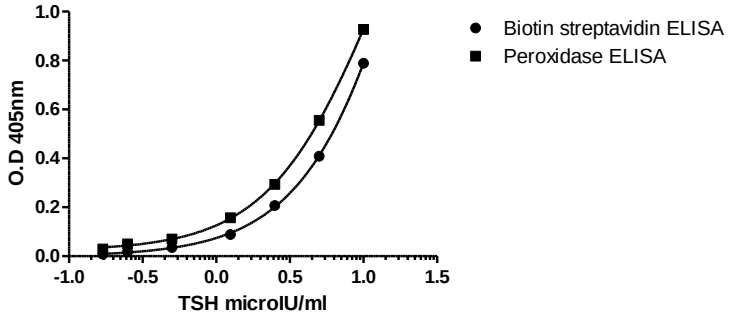

Fig 3: Comparison of Biotin Streptavidin vs Peroxidase ELISA with the introduction of low end calibrators using 4 parameter logistic model

	Biotin Streptavidin	Peroxidase ELISA
Pearson r	0.9996	0.9953
95% confidence interval	0.9979 to 0.9999	0.9732 to 0.9992
P value (two-tailed)	< 0.0001	< 0.0001
Is the correlation significant? (alpha=0.05	Yes	Yes
R square	0.9993	0.9906

Table 5: Correlation analysis of the Biotin Streptavidin vs Peroxidase ELISA with 2 hr incubation with low end calibrators

Previous Patient 1-7 were rerun in this study and the unknown concentrations were interpolated. Both the calibration curves of the two methods were subjected to non linear regression analysis using the cubic spline function and the results obtained are tabulated below.

Patient Sample	Streptavidin	Interpolated concentration	Peroxidase	Interpolated concentration
Unknown sample 1	0.1390	1.788	0.198	1.567
Unknown sample 2	0.1330	1.725	0.204	1.618
Unknown sample 3	0.1080	1.460	0.149	1.194
Unknown sample 4	0.0190	0.258	0.031	0.174
Unknown sample 5	0.0440	0.667	0.066	0.380
Unknown sample 6	0.0070	0.160	0.013	0.086

Unknown sample 7	0.1480	1.882	0.207	1.644

Table 6: Comparative Interpolation of results of patient samples with low working range of calibrators using the cubic spline model.

As observed from the above data there is a very significant ($p<0.05$) signal enhancement of low end values with the streptavidin ELISA as compared to peroxidase ELISA. The value of Sample no 5 which was read as a hyperthyroid case as per peroxidase ELISA was however read as a normal borderline case in streptavidin ELISA as the T3/T4 values matched the record confirming the accuracy and precision of the newly defined calibration curve by the biotin streptavidin method.

IV. Conflict of interest statement

The authors declare that there are no conflicts of interest.

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