Tiroid Fonksiyon Testlerinde Çapraz Reaksiyon: Üçüncü Basamak Merkez Deneyimi

Cross-Reactivity in Thyroid Function Tests: A Tertiary Care Centre Experience

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ÖZET

Amaç: İmmünolojik tekniklerde çapraz reaktivite değişkenlik gösterir ve sıklık çalışmanın gerçekleştirildiği popülasyona, araştırmacının kullandığı yönteme ve reaksiyonu tespit etme tekniğine ve bağlıdır. Bu çalışmada, tiroid fonksiyon testlerinde görülen çapraz reaktivitenin değerlendirilmesi amaçlanmıştır.

Gereç ve yöntem: Sekiz aylık sürede 47915 hastanın TSH, serbest T3 ve serbest T4 testleri boyunca analiz edildi. Şüpheli beş örnek DXI800 (Beckman Coulter, ABD) cihazında analiz edilmiş ve Cobas e601 (Roche Diagnostics, Almanya) alternatif bir cihaz olarak kullanılmıştır. Ayrıca TSH için seri dilüsyon, polietilen glikol (PEG) ile presipitasyon ve heterofilik bloke edici tüplerle (HBT, Scantibodies Laboratuvarı) inkübasyon gerçekleştirilmiştir.

Bulgular: İki farklı immunoassay ile çalışılan testlerin sonuçları desirable bias ve total izin verilen hataya göre değerlendirildiğinde limitlerin üzerinde çıkmıştır (TSH için %7,8 ve %23,7, serbest T3 için %4,8 ve %11,3, serbest T4 için %3,3 ve %8). HBT ile inkübasyondan sonra, geri kazanım oranları tüm numuneler için %50'nin üzerinde bulunmuştur. Seri dilüsyonda doğrusal eğriler gözlenmiştir. PEG presipitasyonundan sonra; sadece bir numunede %40'ın altında geri kazanım elde edilmiştir.

Sonuç: Bu çalışmada, 47915 hastada 5 şüpheli örnek değerlendirilmiştir ve interferans düşündüren bir örnek tespit edilmiştir. Literatürden farklı sıklık (% 0,05 ila% 6) bulunmuş olmasının, hasta popülasyonunda, kullanılan immünolojik yöntemde ve gözlem süresindeki varyasyondan kaynaklanıyor olabileceği düşünülmüştür.

Anahtar kelimeler: immün yöntem, interferans, tiroid fonksiyon testleri, çapraz reaksiyon, heterofilik bloke edici tüp

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ABSTRACT

Aim: Cross-reactivity in immunological techniques varies and the frequency depends on the population of the study, technique for detecting the reaction and researcher's method. In this study our aim was to evaluate the cross-reactivity in thyroid function tests.

Material and Methods: TSH, free T3 and freeT4 tests of 47915 patients were analyzed for eight months. Suspected five samples were analyzed with DXI800 (Beckman Coulter, USA) and Cobas e601(Roche Diagnostics, Germany) was used as an alternative device. Also serial dilution, polyethylene glycol (PEG) precipitation and incubation with heterophilic blocking tubes (HBT, Scantibodies Laboratory) were performed for TSH.

Results: The results of the tests performed on two different immunoassays were above the limits when evaluated according to desirable bias and total allowable error (7.8% ve 23.7% for TSH, 4.8% ve 11.3% for free T3, 3.3% ve 8% for free T4). After incubation with HBT, recovery rates were above 50% for all samples. Linear curves had observed in serial dilution. After PEG precipitation; below 40% of recovery had obtained in one sample, therefore it was defined as macro-TSH.

Conclusion: In the current study, we evaluated 5 suspected samples in 47915 and found one sample considering interference. The difference of frequency from the literature (0.05% to 6%) may be due to the variation in patient population, the immunological method used, and the observation period.

Keywords: immunoassay, interference, thyroid function tests, cross-reactivity, heterophilic blocking tube

INTRODUCTION

Laboratory results play an important role in clinical decision making processes, and laboratories use extensive quality control procedures designed to minimize the number of errors. Immunoassays are used in clinical laboratories to monitor hormones, tumor markers, antibodies against infectious or allergenic agents, analysis of serum proteins and therapeutic drugs. Immunoassays show high sensitivity and a wide detection range(1) .

Although immunoassays are widely used today, there are some limitations. The limited specificity of the binding molecule may cause cross-reactivity with the substances with similar binding domains which react like the analyte. The presence of bilirubin, hemoglobin or lipid in the sample can affect signal formation, resulting in incorrect results (1, 2). Very high antigen levels, depending on the antibody concentrations of the reagent used in the assay, can often reduce the level by forming single antigen-antibody complexes instead of signal-forming sandwich complexes. This is known as 'Hook effect' and Hook effect is one of the false negative causes (3). Besides, the usage of anti-animal monoclonal antibodies for therapy or imaging increases the possibility of immunoassay interference (2). There are examples of human anti-mouse antibody interference reported for numerous analytes (2, 4-9). Endogenous human antibodies (such as autoantibodies, immunoglobulins, rheumatoid factors) in the sample can interact with reagent components used in the method and cause heterophilic interference and false positive or negative results(10). According to the literature, the studies indicated that the frequency of the cross reaction for different immunoassay platforms changed from 0.05% to 6% (11-15).

Serum thyroid hormone levels measured by immunoassays are the most commonly used tests in diagnosis, follow-up and treatment of thyroid diseases. Macro-TSH is a macromolecule formed by the combination of autoimmune anti-TSH Ig and TSH molecules. TSH is a small molecule excreted from kidneys. Renal clearence markedly reduces in concern with the presence of macro-TSH causing false high TSH concentrations measured by immunoassay techniques (16, 17). When a higher TSH result is seen in a patient, it can indicate future thyroid disease (with a family history or presence of autoantibodies or use of drugs etc.(18, 19). Unfavorable changes compared with the

patient's previous results and in cases where the test result is incompatible with the clinical diagnosis according to the clinician's opinion may suggest the possibility of a cross-reaction. Also the possibility of cross-reactivity could be suspected if there is a disproportionate change in thyroid stimulating hormone (TSH) with serum free triiodothyronine (fT3) and free thyroxine (fT4) levels or low serum fT4 levels with normal TSH levels in drug-free and out-patients or there is a history of exposure to animal antibodies (19).

When an incompatible result is suspected, there are a few procedures that can be performed to verify the interference: serial dilution of the sample, the analysis of the sample with different immunoassay using different reagents or antibodies, re-analysis of the sample after precipitation and removal of high molecular weight proteins, re-analysis of the sample after removal of interfering agents using blocking reagents or preincubation of the patient sera with commercially available neutralizing agents of high dose of mouse immunoglobulin (11, 19).

In this study we aimed to investigate the interference in TSH, fT3 and fT4 results in Beckman Coulter DXI 800 for a period of 8 months and evaluate the causes of the suspected cross-reactivity.

MATERIALS AND METHODS

The study was conducted prospectively. 47915 patient samples were included in the study for eight month period with requested TSH, fT3 and fT4 tests. Suspected result was determined according to the clinician's statement about the patient's unexpected or discordant result with the clinical picture. Clinicians were informed before the study to call the laboratorians for any unexpected or discordant results of thyroid function test results. The study was approved by local ethical committee (2014-909).

4 procedures were performed for the suspected samples of cross reactivity after the laboratory evaluation and communications with clinicians.

- Analysing the sample with an alternative immunoassay technique using different reagents
- The linearity study (serial dilutions)
- Re-analysing the sample after the precipitation of high molecular weight proteins with polyethylene glycol
- Re-analysing the sample after blocking the interfering agent

Immunoassay technique in the laboratory

Serum TSH levels were analysed with Access HYPER sensitive hTSH assay kit (Third generation) in a Beckman Coulter DXI 800 (California, USA) autoanalyser using non-competetive paramagnetic particle, chemiluminescent immunoassay technique. The expected values for TSH levels were 0.34-5.60 μIU/mL. fT3 and fT4 levels were analysed with Access Free T3 ve Access Free T4 assay reagents in a Beckman Coulter DXI 800 (California, USA) autoanalyser using competetive paramagnetic particle, chemiluminescent immunoassay technique. Anti-mouse goat antibody was used for TSH assay, anti-goat, anti-bovine and anti-bird antibodies were used for fT3 assay, anti-bird and anti-murine antibodies were used for fT4 assay. The expected values were 2.5-3.9 pg/mL for fT3 assay and 0.61 - 1.12 ng/dL for fT4 assay. Intra and inter-assay coefficient of variation (CV) values were 4.4% and 8.08% for fT4 assay, 6.6% and 8.0% for fT3 assay and 5.83% and 8.88% for TSH assay, respectively.

Alternative immunoassay technique

Serum TSH levels were analysed with Elecsys TSH assay kit (Third generation) in a Roche Diagnostics Cobas e601 (Mannheim, Germany) autoanalyser using a non-competetive electrochemiluminescent immunoassay (ECLIA) technique. The expected values for TSH levels were 0.27–4.2 μIU/mL. fT3 and fT4 levels were analysed with Elecsys FT3 III

and Elecsys FT4 II assay kit in a Roche Diagnostics Cobas e601 (Mannheim, Germany) autoanalyser using a competetive electrochemiluminescent immunoassay (ECLIA) technique. Anti-mouse antibody was used for TSH assay, anti-sheep antibody was used for fT3 assay, anti-sheep antibody was used for fT4 assay. The expected values were 2.0-4.4 pg/mL for fT3 assay and 0.93-1.7 ng/dL for fT4 assay. Intra and inter-assay coefficient of variation (CV) values were 4.3% and 8.4% for fT4 assay, 6.5% and 7.2 % for fT3 assay and 3.0% and 7.2% for TSH assay, respectively.

Serial dilution

Serial dilutions of patient sera for TSH values were performed in proportions of 1/2, 1/4, 1/8 and 1/16 using Sample Diluent A (Beckman Coulter, USA) as recommended by the manufacturer. TSH levels were simultaneously analyzed in DXI 800 (Beckman Coulter, USA) autoanalyzer. The lack of linear slope in the sample results evaluated by serial dilution may be evaluated as positive in terms of cross reaction. The results of serial dilutions in immunoassay tests may vary from perfect linear to non-linear response different from conventional biochemical assays (20, 21).

PEG precipitation

2.5 g of PEG 6000 (Merck Schuchardt, Germany) was dissolved in 10 mL of distilled water. The patient sera and 25% PEG solution were mixed in equal volumes and centrifuged at 250 × *g* for 30 minutes. After centrifugation, the supernatant was removed and TSH levels were measured in Beckman Coulter DXI 800 autoanalyzer. Recovery was calculated according to the formula: [2 x TSH (after mixing with PEG) / TSH (before mixing with PEG) x 100]. Recovery below 40% was considered to indicate the presence of high molecular weight proteins (22).

Heterophilic blocking tube

Heterophilic blocking tube (HBT) (Scantibodies Laboratory, CA, ABD) was used to eliminate false positive heterophilic interference. The sample was thoroughly mixed with the reagent in HBT and incubated at room temperature (18-28°C) for one hour and treated sample was analysed. The recovery was calculated according to the formula [TSH (after incubation with HBT) / TSH (before incubation with HBT) × 100]. Recovery below 50% was considered to indicate the presence of heterophilic antibodies (23).

Statistical analysis

Statistical data was processed using MedCalc Software v12.3.0.0 (Ostende, Belgium) and Statistical Package for Social Sciences for Windows (SPSS v18). The conformity of variables to normal distribution was tested with Shapiro-Wilk test. Microsoft Office Excel 2007 was used for calculations. The biases for TSH, fT4 and fT3 from two different immunoassays were compared with the current desirable allowable bias and total allowable error (TEa) based on biological variation (24). The mean difference for each test (TSH, fT4 and fT3) was calculated according to the formula: mean difference (%) = [(first immunoassay test mean - second immunoassay mean) / first immunoassay test mean x 100]. Passing–Bablok regression analysis was performed. Bias and %95 limits of agreement assessments were performed using Bland-Altman analysis.

RESULTS

For eight month period, 47915 results of patients were evaluated and 5 suspected results were re-evaluated. Four patients were female and one patient was a male and ages of the patients were 26, 34, 58, 25 and 62 years, respectively. The mean bias of two different immunoassay TSH, fT3 and fT4 results of 5 incompatible patient samples were calculated and clinical significance level was compared according to desirable bias and TEa (Table 1) (clinical significance was compared with the current desirable allowable bias data based on biological

variation) (24). Desirable bias and TEa values for TSH were %7.8 ve %23.7, Desirable bias and TEa values for fT3 were %4.8 ve 11.3 and Desirable bias and TEa values for fT4 were %3.3 ve %8. For TSH test, the mean bias of one of the 5 patient samples was lower (4.25%) than the desirable bias (7.8%), and the mean bias of the other 4 patient samples were found to be clinically significantly higher than the desirable bias. Also, the mean bias results of 3 patient samples exceed the TEa limit. All of the calculated mean bias results for fT3 were clinically significantly higher than desirable bias and the mean bias of 4 patient samples also exceed the TEa limit. For fT4 test, the mean bias results of 4 patient samples except one (1.23%) were significantly higher than desirable bias (3.3%) and exceed TEa limit.

Passing–Bablok regression analysis yielded the equation "y = -0.125 + 1.18x" between two immunoassay systems. TSH results of two immunoasssays showed very strong correlation (r=0.90 p=0.04). According to Bland-Altman analysis, TSH results showed a mean bias of 0.4 (95% limit of agreement between -8.6 and 9.5) (Figure 1 and 2).

Serial dilutions of patient sera for TSH values were performed in proportions of 1/2, 1/4, 1/8 and 1/16. The results of serial dilutions of five patients' sera showed linear slope.

2.5% PEG 6000 solution was mixed with patient's serum in order to precipitate high molecular weight proteins. Recovery below 40% was obtained from only one of five patients sera (Table 2).

Samples were mixed with the reagent in HBTs and reanalysed after incubation for one hour. TSH values of the patients before and after the incubation were showed in Table 2. Recovery below 50% was considered to be positive in terms of heterophilic antibodies. All of the patients had recovery over 50%.

Table 1. TSH, fT3 and fT4 test results performed with two different immunoassays

	TSH[1] 0.34-5.6 (μIU/mL)	TSH[2] 0.27-4.2 (μIU/mL)	Mean Bias % (TSH)	fT3[1] 2.5-3.9 (pg/mL)	fT3[2] 2.0-4.4 (pg/mL)	Mean Bias % (fT3)	fT4[1] 0.61-1.12 (ng/dL)	fT4[2] 0.93-1.7 (ng/dL)	Mean Bias % (fT4)
P1	2.08	2.33	12.01[d]	4.22	4.64	9.95[d]	2.01	2.39	18.90[d,t]
P2	23.01	22.03	4.25	2.7	2.14	20.74[d]	0.49	0.73	48.97[d,t]
P3	2.45	3.3	34[d,t]	3.92	5.62	43.36[d,t]	1.45	2.02	39.31[d,t]
P4	9.4	16.82	73.61[d,t]	4.29	2.98	30.53[d,t]	0.63	0.51	19.04[d,t]
P5	12.49	7.1	43.15[d,t]	5.94	4.92	17.17[d]	0.81	0.82	1.23

1: Beckman Coulter DXI800, 2: Roche Cobas e601, d:above desirable bias limits, t: above total allowable bias limits, Desirable Bias and TEa for TSH: %7.8 ve %23.7, Desirable Bias and TEa for fT3: %4.8 ve 11.3, Desirable Bias and TEa for fT4: %3.3 ve %8

Table 2. TSH results after PEG precipitation and incubation with HBT

	TSH (μIU/mL)	TSH after PEG precipitation (μIU/mL)	Recovery (%)	TSH after HBT incubation (μIU/mL)	Recovery (%)
P1	2.08	12.34	53.6	23.13	100.5
P2	23.01	2.33	95.1	2.34	95.5
P3	2.45	1.8	86.5	1.81	87
P4	9.4	3.61	38.4	7.35	78.1
P5	12.49	7.01	56.5	11.23	89.9

PEG: polyethylene glycol, HBT: Heterophilic blocking tube

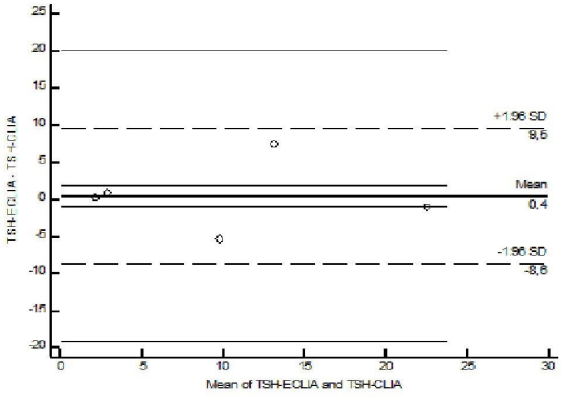

Figure 1. Bland-Altman graph shows the difference between the mean TSH values studied by two different methods

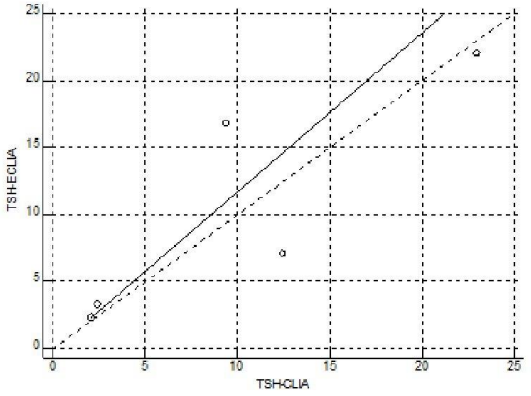

Figure 2. Comparison of two different methods used in the measurement of TSH (y = -0.125 + 1.18x) (Passing-Bablok Regression Analysis)

DISCUSSION

In the current study, 5 suspected samples were evaluated for cross reactivity in terms of TSH, fT4 and fT3 results. We performed four different procedures in order to show cross reactivity. The results of the tests performed on two different immunoassays were found to be above the limits when evaluated according to desirable bias and TEa. Also one of the patients' samples was found to have high molecular weight protein.

The discordant results of the samples indicated the presence of interfering substances that we could not differentiate in two different immunoassay devices. It is indicated that the difference could be originated from the immune complex separation methodology of each manufacturer (25). Analysing suspicious samples with alternative immunoassay technique to exclude the cross-reaction resulting from the antibody that may occur against one or more of the reagents in the method used is one of the recommended practices (10, 12, 20, 21, 26). However, according to the literature, there is no single test to exclude cross-reaction and opinions about whether the results of two different immunological methods which are similar or different can exclude or accept a cross-reaction (13, 14, 27-29).

The second procedure was testing linearity with serial dilutions of the patient samples using recommended diluent. The concentration of reactive antibodies in the test mixture is always constant, but the concentration of the antibodies that cause cross-reaction change from one patient to another. Dilution of samples cause to change the relative ratio of reactive and cross-reactive antibodies (30). In our study, serial dilution of five patients' sera resulted linear slopes. The results indicated that there was no cross reacting agents in the sera. However, serial dilution alone does not provide any definitive information about the cross-reaction. So, in the case of a cross-reaction, there is also a possibility of obtaining a linear response of serial dilution (16, 31). In the literature, there are studies indicating that the results of serial dilutions may vary in spite of the presence of cross reaction (16, 17, 27, 32). In a study, it was indicated that in linearity studies only 60% of the cross reactive samples may result in non-linear slopes (20).

The third procedure was reanalysing the samples after the precipitation of high molecular weight proteins with PEG. Only one of the samples resulted recovery below 40%. It is considered to show the presence of high molecular weight proteins formed macro-TSH. However, some researchers have evaluated the result of <20% recovery rate positively in terms of macroTSH as in controls the recovery rate was obtained between 58-79% and 61.7±10.3%, but in

the studies it is also recommended that each laboratory should set their cut-off value (17, 33-36).

Based on these studies, the only sample we accept as positive is considered to be negative. In the routine use of our laboratory, we prefer below 40% recovery rate, representing the presence of macro-TSH. Our patient has 762.1 IU/mL (0-9) Anti-TPO (anti-thyroid peroxidase antibody) concentration. As we thought that the patient has macro-TSH, our PEG precipitation finding supports this result. Also our patient had subclinical hypothyroidism with the presence of macro-TSH. In studies, authors also stated that macro TSH may be common in patients with subclinical hypothyroidism and the etiology of macro TSH seems to be heterogeneous (34, 35, 37). In a study, patients with subclinical hypothyroidism were evaluated and macro-TSH was present 0.79% of the patients. Authors indicated that the nature of reagent antibodies, heterogenous nature of macro-TSH leads variable detectability by different immunoassay platforms (35). Heterophilic antibodies, thyroid hormone autoantibodies, and assay specific interference are indicated as the possible causes of macro-TSH (38). The presence of macro-TSH is confirmed by PEG precipitation and gel filtration chromatography (GFC). GFC is described as gold standard technique for confirmation, however, this method is expensive, labor intensive and is not routinely used in many clinical laboratories (17). Many studies compared the results of PEG precipitation technique and GFC for suspicious samples in terms of macro-TSH and indicated to use PEG precipitation technique for first choice in order to show the presence of macromolecules as it is rapid, cost effective and easily achievable in many routine clinical laboratories (16, 39-43).

The fourth and the last procedure was reanalysing the sample after blocking the interfering agents. The results of the samples were compared before and after incubation with HBTs. After incubation with HBT, recovery rates were above 50% for all samples. Accordingly, it was considered that the inconsistency in patient results did not originate from heterophile antibodies. Interfering antibodies can be defined as heterophiles if there is no well-defined immunogen or medical therapy with animal immunoglobulins, or if they react with immunoglobulins of two or more species (multispecific) or exhibit rheumatoid factor activity (42). With the development of monoclonal antibody hybridization technology in the 1980s, Kaplan and Levinson have noted that the use of many immunometric method kit formats can lead to an increase in the number of reports reported by bridging interferences, and the determination of the structure of the antibody together with the steps could reduce interference (42). Low-affinity human IgM antibodies reach the steady-state in the reaction for a long time, and their separation is at a lower rate. This may explain that the washing steps can not remove the interfering antibodies. Ismail et al. indicated that shorter incubation times may lead to interferences (44). In some studies evaluating incompatible TSH results with HBT incubation, the results were not significantly different from preincubation results indicating absence of heterophile antibodies (17, 27, 29, 33, 43, 45). However, manufacturers state that the tubes contain only spesific blocking agents, so that unsignificant results of the studies may not exclude the possibility of cross-reaction arising from other antibodies. There are also studies indicating that the values obtained before and after HBT incubation are highly likely to be due to heterophile antibody-induced cross-reactivity (15, 30, 32, 46, 47).

CONCLUSION

In our study, we evaluated 5 suspected samples in 47915 and found one sample considering interference. The difference from the literature (0.05% to 6%) may be due to the variation in patient population, the immunological method used, and the observation period. Establishing permanent records in the files for patients with interference detected in immunoassay results will be useful for evaluating future immunoassays. Also it is important to know

that cross-reaction can not be predicted and can affect more than one test in the same patient.

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