Smyrna Tıp Dergisi **Araştırma Makalesi**

Evaluation of Signal Cut-off Ratio with Fourth Generation HIV Ag/Ab Screening Test for the Diagnosis of HIV Infection
HIV Enfeksiyonunun Tanısında Dördüncü Nesil HIV Ag/Ab Tarama Testi ile Sinyal Cut-Off Oranının Değerlendirilmesi

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Summary
Objective: It is crucial to confirm the diagnosis via fast and effective assay methods for the effective management of human immunodeficiency virus (HIV) infection. This is also important for the initiation of anti-retroviral therapy and prevention of HIV transmission. In this study, it was aimed to elucidate the accuracy of S/CO value obtained by the fourth generation enzyme immunoassay (EIA) test by comparing its relationship with false positivity rate.
Material and Method: A total of 126 cases who were repeatedly reactive by anti-HIV ELISA test result with confirmed HIV infection have been enrolled in this retrospective analysis. The serum and plasma samples that were repeatedly reactive for anti-HIV antibodies by micro-ELISA method and were confirmed by line immunoassay (LIA) method. In addition, if samples were negative or indeterminate by LIA test, quantitative real time reverse transcriptase polymerase chain reaction (q–RT–PCR) has been performed.
Results: HIV–1 infection was confirmed by LIA and /or molecular method in 88 (69.8%) of cases and their gender distribution was; 77 (87.5%) male and 11 (28.9%) female. Additionally, 38 cases who were positive by ELISA LIA and/or RT–PCR was negative for HIV. Thus false positive results were detected in 30.1% of cases by ELISA test. These cases had a S/CO index of ≥1. False positivity decreased with increasing S/CO value. False positivity rate was found to be 12.5% (n=11) in males and 71.1% (n=27) in females ($p<0.001$). The sensitivity, specificity, positive and negative predictive value were 100%, 95%, 97%, 100%, respectively, when the S/CO value was 7.20 by ROC analysis.
Conclusion: Since HIV prevalence of the population affects the positive predictive value of the test and can cause false positive results, each laboratory should determine the optimal S/CO value that increases the sensitivity and specificity of the test to avoid adverse situations.
Key words: ELISA, HIV, PCR, predictive ROC analysis

Özet
Amaç: İnsan immün yetmezlik virüsü (HIV) enfeksiyonunun etkin yönetimi için tanının hızlı ve etkili tahlil yöntemleri ile doğrulanması çok önemlidir (1). Bu, anti-retroviral tedavinin başlatılması ve HIV bulaşmasının önlenmesi için de önemlidir. Bu çalışmada dördüncü kuşak enzim immunoassay (EIA) testi ile elde edilen S/CO değerinin yanlış pozitiflik oranı ile ilişkisini karşılaştırarak doğruluğunu aydınlatmak amaçlanmıştır.
Gereç ve Yöntem: Bu retrospektif analize, anti-HIV ELISA test sonucu ile doğrulanmış HIV enfeksiyonu ile tekrar tekrar reaktif olan toplam 126 vaka dahil edilmiştir. Mikro-ELISA yöntemi ile anti-HIV antikorları için tekrar tekrar reaktif olan serum ve plazma örnekleri, line immunoassay (LIA) yöntemi ile doğrulandı. Ayrıca LIA testi ile numuneler negatif veya belirsiz ise kantitatif gerçek zamanlı ters transkriptaz polimeraz zincir reaksiyonu (q–RT–PCR) yapılmıştır.

Bulgular: Vakaların 88'inde (%69,8) LIA ve/veya moleküler yöntemle HIV–1 enfeksiyonu doğrulandı ve cinsiyet dağılımı 77 (%87,5) erkek, 11 (%28,9) kadın olarak belirlendi. Ek olarak, ELISA LIA ve/veya RT – PCR ile pozitif olan 38 vaka HIV için negatifti. Böylece ELISA testi ile vakaların %30,1'inde yanlış pozitif sonuçlar tespit edilmiştir. Bu vakaların S/CO indeksi ≥ 1'di. S/CO değeri arttıkça yanlış pozitiflik azaldı. Yalancı pozitiflik oranı erkeklerde %12,5 (n=11), kadınlarda %71,1 (n=27) olarak bulundu (p<0,001). ROC analizi ile S/CO değeri 7,20 olduğunda sensitivite, spesifite, pozitif ve negatif prediktif değer sırasıyla %100, %95, %97, %100 idi.
Sonuç: Toplumdaki HIV prevalansı, testin pozitif prediktif değerini etkilediğinden ve yanlış pozitif sonuçlara neden olabileceğinden, olumsuz durumlardan kaçınmak için her laboratuvar testin duyarlılığını ve özgüllüğünü artıran optimal S/CO değerini belirlemelidir.
Anahtar kelimeler: ELISA, HIV, PCR, prediktif ROC analizi

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Introduction

It is crucial to confirm the diagnosis via fast and effective assay methods for the effective management of human immunodeficiency virus (HIV) infection (1). Early and rapid detection of HIV infection is important for the initiation of anti-retroviral therapy and prevention of HIV transmission. There were 29284 HIV positive and 2052 acquired immunodeficiency syndrome (AIDS) cases reported from 1985 to 15 November 2021 in Turkey (2).

Technological innovations and new guidelines have enabled the identification of HIV in the early period (3,4). While the first, second and third generation HIV assays were based on antibody determination, the fourth generation HIV assay has the ability to detect both antibody and antigen presence (5,6). Rapid and accurate diagnosis at early stage plays an important role in preventing the transmission of HIV and the initiation of treatment (7,8).

The Center for Disease Control and Prevention (CDC) recommends a two-step diagnostic algorithm in the diagnosis of HIV with the use of a 4th generation ELISA test based on the detection of HIV-1 p24 antigen and HIV-1/2 antibody (IgM and IgG) as the first step. This should be followed by the second test, that can distinguish HIV–1 and HIV–2 infection (9,10). Although the fourth generation ELISA tests shortens the window period, false positive results are obtained and confirmatory tests are needed (3,4). The western blot (WB) test is a method commonly used for the confirmation of HIV screening tests since 1997. However, the WB test has several disadvantages, such as: labour intensive low sensitivity, frequently generating indeterminate results and it is expensive (11). It is recommended that the nucleic acid amplification test may be applied to samples that are reactive or indeterminate by a confirmatory test (12,13).

The 5th generation HIV screening test, which has been licensed by the FDA in 2015 is not routinely used in our country although they have the ability to detect separately HIV antibodies and p24 antigen (5). In addition, ELISA tests may show false positive results due to low prevalence of infection, cross-reactions and molecular mimicking, recent influenza, hepatitis B and rabies vaccination, viral infections, auto-immune disease, renal failure, hemodialysis and pregnancy (14,15,16,17).

There are limited studies using signal/cut-off (S/CO) rates in screening tests as a predictor in the diagnosis of HIV infection (18). It is reported that S/CO ratio can be utilized in the clinical decision-making process in case it can predict the diagnosis of HIV before a confirmatory test (19).

In this study, it was aimed to elucidate the accuracy of S/CO value obtained by the fourth generation enzyme immunoassay (EIA) test by comparing its relationship with false positivity rate.

Material and Methods

A total of 126 cases who were repeatedly reactive by anti-HIV ELISA test result with confirmed HIV infection between February 2016 and December 2018 have been enrolled in this retrospective analysis. The ethics committee approval has been granted with protocol number: 2019/49/133. The study complied with the Declaration of Helsinki and informed consent has been obtained from all participants.

The study included serum and plasma samples that were repeatedly reactive for anti-HIV antibodies by micro-ELISA method and were confirmed by line immunoassay (LIA) method. In addition, if samples were negative or indeterminate by LIA test, quantitative real time reverse transcriptase polymerase chain reaction (q – RT – PCR) has been performed for HIV-1 RNA. If HIV-1 RNA above 5.000 copies/ml (10.000 IU/ml) was detected in these samples then they were also included in the study.

Enzyme Immunoassay (EIA)

The anti-HIV Ag/Ab test was performed according to the manufacturer's recommendation with the Grifols™ Triturus®ELISA analyzer using the Siemens Enzygnost® HIV Integral 4 (Marburg, Germany) kit based on the two-step 'sandwich' principle. The Siemens Enzygnost® HIV Integral 4, is a 4th generation ELISA kit that simultaneously can detect HIV p24 antigen and antibodies to HIV–1 and HIV–2 in serum or plasma. The microplate wells in the kit were coated with monoclonal antibodies against HIV–1 and HIV–2 antigens, as well as recombinant HIV–1 and 2 antigens. The results were evaluated according to the manufacturer's recommendation. The cut-off value was calculated by adding 0.180 to the absorbance value of the negative control. Test results was calculated by proportioning the sample absorbance to the cut-off value. Ratio-based assessment was performed as HIV-negative: index <1 and HIV-reactive: index ≥1. Anti-HIV test reactive-sample was runtwice from the same blood sample and test was repeated with a new blood sample as well. When at least two assay results were positive the sample was labeled as repeatedly reactive.

Line immunoassay (LIA)

Serum samples with recurrent reactivity were confirmed by LIA method using the anti-HIV LIA assay kit (INNO-LIA™ HIV I / II Score, Belgium). LIA has been utilized for the confirmation and discrimination of antibodies to HIV–1, HIV–1 group O and HIV–2 in human serum and plasma.

Since false-positive reactions have frequently been observed with current ELISA tests, it was strongly recommended to confirm repeatedly reactive samples by use of other reliable techniques such as the INNO-LIA HIV I/II Score. Recombinant proteins and synthetic peptide from HIV–1 (sgp120, gp41, p31, p24 and p17) and HIV–2 (gp36 and sgp105) , and a synthetic peptide from HIV–1 group O, are coated as discrete lines on a nylon strip with plastic backing. The results were evaluated as negative, indetermine and positive according to the manufacturer's recommendation.

Isolation of HIV-1 RNA and RT – PCR

HIV–1 RNA isolation was performed on the QIAsymphony SP/AS instrument (Qiagen GmbH, Germany) instrument using the HIV–1 RNA isolation kit (Qiagen, Germany). The product obtained after isolation was amplified on the ROTOR-GENE Q instrument (Qiagen GmbH, Germany) using the Artus HIV–1 QS-RGQ Kit (Qiagen).

Statistical Analysis

Data analysis was performed using SPSS 21 (SPSS Inc, Chicago, IL, USA) program. The suitability of the variables to normal distribution was examined by visual methods and Kolmogorov-Smirnov test. Variables were compared using Student's t test or Mann-Whitney U test and qualitative variables were compared using Pearson Chi-Square or Fisher exact tests. HIV confirmation by LIA and detection of HIV-RNA by real-time PCR were accepted as the gold standard diagnostic method. The performance of anti-HIV test in predicting viremia was evaluated by ROC analysis. Sensitivity, specificity, negative predictive values and positive predictive values were investigated by determining the significant cut-off values of the test with ROC curve analysis. P values less than 0.05 were considered statistically significant.

Results

A total of 126 cases and 44683 have been included in this study. Majority of these cases were male 88 (69.8%) and 38 (31.2%) were female. The mean age of males was 41.57±14.55 years (ranging between 14–73) and females was 41.82±14.51 years (ranging between 16–76) respectively (p=0.93). The mean age of the confirmed HIV–1 infected male and female patients was 40.58±13.87 years (ranging between 20–73) and 37.64±11.71 years (ranging between 16–58) respectively (p=0.07).

HIV–1 infection was confirmed by LIA and /or molecular method in 88 (69.8%) of cases and their gender distribution was as follows:

77 (87.5%) male and 11 (28.9%) female. Additionally, 38 cases who were positive by ELISA LIA and/or RT–PCR was negative for HIV. Thus false positive results were detected in 30.1% of cases by ELISA test. These cases had a S/CO index of ≥1. False positivity decreased with increasing S/CO value (Table 1). False positivity rate was found to be 12.5% (n=11) in males and 71.1% (n=27) in females (p<0.001).

The median value of the anti-HIV index of 126 cases was 12 (ranging between 1.10–15.90). Anti-HIV index median values were found to be 1.84 (ranging between 1.10–6.70) and 12.7 (ranging between 3.06–15.90), respectively in patients with HIV–1 negative and positive confirmed by LIA and/or HIV–1 RNA (p<0001). HIV–1 positivity rates were found to be 75.6% (34/45), 72.7% (40/55), 53.8% (14/26) in the <35, 35–54 and ≥55 age group, respectively (p=0.13).

HIV–1 RNA and/or LIA was negative in all cases with anti-HIV index value <3.05 (n=29, 23%), and positive in all cases with anti-HIV index value ≥7.20 (n=86, 68.3%). While HIV–1 positivity was not detected in 97.1% of cases with anti-HIV index values between 1–4 , HIV–1 negativity was not detected in any of the subjects with index value ≥10 (p<0.001) (Table 2).

The sensitivity, specificity, positive and negative predictive value were 100%, 95%, 97%, 100%, respectively, when the S/CO value was 7.20 by ROC analysis. On the other hand, the area under the ROC curve was AUC: 0.99 (95% Cl: 0.99-1.00) and was statistically significant (p <0.001) (Figure 1).

Table 1. *HIV diagnostic results according to signal/cut-off ratio(S/CO) range*

	S/CO values			
	≥1	>3.05	>5.90	>7.20
False positivity n (%)	38 (30.1)	9 (9.27)	1 (1.1)	0
Sensitivity n (%)	*	100 (94.78-100)	98.86 (92.94-99.94)	100 (94.67-100)
Specificity n (%)	*	76.31 (59.38-87.97)	97.36 (85.56-99.86)	95 (81.79-99.12)
Positive predictive value n (%)	*	90.72 (82.67-95.40)	98.86 (92.94-99.94)	97.72 (91.25-99.60)
Negative Predictive valuen (%)	*	100 (85.43-100)	97.36 (84.56-99.86)	100 (88.56-100)

n, number of serum samples, *******Since the confirmatory test was not performed on all serum samples, it could not be calculated.*

Table 2. *Distribution rates of HIV-1 infection in groups of anti-HIV index values*

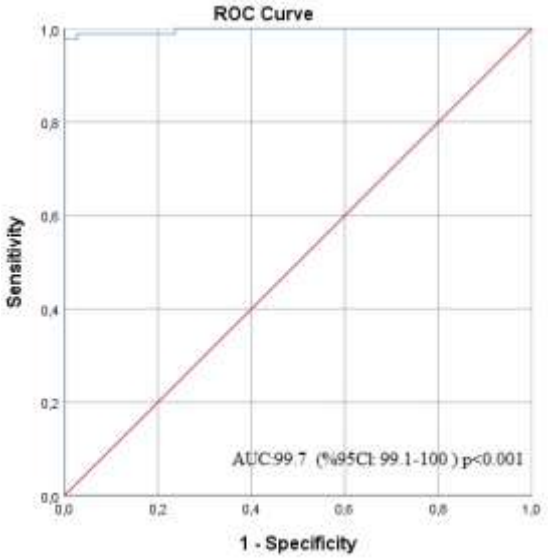
	Groups of anti-HIV index values					
	1-4 n (%)	**5-9 n (%)**	**10-14 n (%)**	**≥15 n (%)**	**Total n (%)**	**p values**
Gender						
Male						**<0.001**
HIV-1 negative	10 (90.9)	1 (14.3)	0 (0)	0 (0)	11 (12.5)	
HIV-1 positive	1 (9.1)	6 (85.7)	61 (100)	9 (100)	77 (87.5)	
Female						**<0.001**
HIV-1 negative	24 (100)	3 (42.9)	0 (0)	0 (0)	27 (71.1)	
HIV-1 positive	0 (0)	4 (57.1)	6 (100)	1(100)	11 (28.9)	
Total						
HIV-1 negative	34 (97.1)	4 (28.6)	0 (0)	0 (0)	38 (30.2)	**<0.001**
HIV-1 positive	1(2.9)	10 (71.4)	67 (100)	10 (100)	88 (69.8)	

n, number of serum samples

Discussion

The performance of HIV infection screening tests has continuously improved since their first use in 1985 (20). The window period is shortened by 4th generation ELISA kits. Although it is more effective in identifying HIV-infected patients, the rate of false positivity is quite low in populations with low HIV prevalence (21).

Figure 1. *Receiver-operating characteristics (ROC) curve of anti-HIV S/CO ratio forpredicting the results of HIV RNA/HIV LIA testing in 126 patients positive for anti-HIV*



False positive results may be observed in influenza or hepatitis B vaccines, viral infections, autoimmune diseases, renal failure, blood transfusion, liver disease, hemodialysis, multiple pregnancies or early pregnancy (16,22). In addition, false positive results can cause serious psychological problems in patients awaiting HIV confirmation test results (23). In the present study, the reasons for false positivity were excluded from the scope of the study because of the retrospective analysis of laboratory records did not include clinical data.

A high cut-off index value or S/CO ratio is associated with true positivity (15). In a study conducted in Korea, false HIV positivity was reported in 63% of 54 patients who were found to be HIV reactive with the 4th generation ADVIA Centaur HIV Ag/Ab Combo test kit. A total of 34 samples that were found to be false positive and the median value of S/CO was 1.4 (ranging between 1.0 – 12.0) (24).

In Elecsys® method, samples tested for anti-HIV antibody by the commercial kit samples with cut-off index values between 0.91–4.85 (cut-off <0.9) were false reactive, and the confirmation test of all samples with cut-off index value of ≥84.25 was positive. On the contrary, samples tested by another kit ARCHITECT®, with cut-off index values 1.09–12.49 (cut-off <1.0) were found to be the false reactive and all ≥45.65 samples were found to be true positive (25).

In Spain, false positivity was detected in 27 (10.5%) of 256 anti-HIV reactive samples with the Abbott Architect® HIV-Ag/Ab-Combo 4th Generation EIA. According to their results, the false positivity in 19 out of 19 samples the false positivity ratio decreased as the S/CO ratio increased. In a study by Chacón et al. (26), 220 samples with S/CO ratio of >50 were detected to be all true positive. In the presented study, it was confirmed that the presence of HIV–1 infection by LIA and/or molecular method in 88 (69.8%) of 126 cases with anti-HIV reactive detected by Siemens Enzygnost® HIV Integral 4 ELISA kit. HIV–1 positivity was not detected by HIV RNA and/or LIA in all cases with anti-HIV index <3.05. Also, no false positivity in any of the samples with an anti-HIV index of ≥7.2. In the present study, the false positive ratio decreased with increasing S/CO ratio as observed other studies.

False positivity problems of screening tests continue in populations with low HIV prevalence as in T. Determination of cut-off index value in test kits is clinically important for predicting true HIV viremia. In a study conducted using the Abbott HIV Ag/Ab Combo assay in China, the highest sensitivity and specificity (100%, 99.43%) rates were found when the S/CO of the samples was 11.26 and the area under the curve in the ROC curve analysis was found to be 0.998 (27). In a similar study conducted in Korea, the highest sensitivity and specificity (100%, 99.99%) have been achieved when the anti-HIV cut-off value was taken as 6.6 (15).

The main limitation of this study could be attributed to its retrospective nature. The strength of this research lies beneath the fact that it was a rarely performed study reported from an HIV confirmatory center in Turkey.

Conclusion

Since HIV prevalence of the population affects the positive predictive value of the test and can cause false positive results, each laboratory should determine the optimal S/CO value that increases the sensitivity and specificity of the test to avoid adverse situations. Examining the COI values obtained in HIV EIA tests although will not provide a definitive diagnosis but will increase our predictions in the results.

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Competing interests
The authors declare that they have no competing interests.
Ethical Declaration
The ethics committee approval has been granted with protocol number: 116.2017.124. The study complied with the Declaration of Helsinki and informed consent has been obtained from all participants.

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