JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Threshold value of the anti-HCV test in the diagnosis of HCV infection

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Abstract

Introduction: In the diagnosis of hepatitis C virus (HCV) infection, the first step is screening for anti-HCV antibodies, and positive results are generally confirmed with nucleic acid amplification tests. Recent studies have reported that more compatible results have been obtained with the HCV RNA test using signal to cut-off (S/Co) values >1, which are the routine reactivity threshold for the anti-HCV enzyme immunoassay (EIA) test. The aim of this study was to determine the most appropriate S/Co value for the anti-HCV test, predicting HCV infection.

Methodology: Comparisons were made between results of 559 patients who underwent anti-HCV with ECLIA method and HCV RNA tests with real-time polymerase chain reaction (PCR) method. By accepting the HCV-RNA test as the gold standard for HCV infection, the sensitivity, specificity and predictive values of the ECLIA test were determined and statistical "receiver operating characteristic" (ROC) analysis was applied to determine the most appropriate threshold.

Results: Between January 2013 and April 2018, a total of 81,203 serum samples were examined. Of 559 anti-HCV positive patients, HCV RNA positivity was determined in 214 (38.2%). According to the ROC analysis results, the most appropriate S/Co value was determined as 12.27, at which sensitivity was 94.4 %, and specificity 97.4%. The positive and negative predictive values were calculated at the high rate of 95.7% and 96.6% respectively.

Conclusions: The results of this study investigating the anti-HCV reactivity values which could be used in the diagnosis of HCV infection determined the most appropriate value to be 12.27.

Key words: Hepatitis C virus; anti-HCV; S/Co rate; HCV-RNA; sensitivity.

J Infect Dev Ctries 2019; 13(10):914-919. doi:10.3855/jidc.11657

(Received 14 May 2019 – Accepted 20 September 2019)

Introduction

Hepatitis C virus (HCV) infection constitutes one of the most important healthcare problems worldwide. The World Health Organization (WHO) estimates that approximately 1.6% of the global population is infected with HCV [1]. In Turkey this rate varies from 0.3% to 1.8% [2]. Hepatitis C is the leading cause for end-stage liver complications, including hepatocellular carcinoma and the need for liver transplantation; the frequency of these is expected to increase two-to threefold by 2030 [3]. Accurate diagnosis of active HCV infection is important not only because of the associated morbidity and mortality but also because of the possibility of spontaneous or pharmacology-induced sustained virologic cure [4]. The recommended treatments in Turkey for naiv patients are Sofosbuvir + Ledipasvir for 8 weeks for those infected with genotype 1, 4, 5, and 6, and Paritaprevir/ritonavir + Ombitasvir + Dasabuvir for 12 weeks for those infected with genotype 1b [5].

The microbiology laboratory plays a significant role in the diagnosis of HCV infection. The first stage in diagnosis is the determination of anti-HCV antibodies with enzyme immunoassay (EIA) or the chemiluminescence immunoassay (CIA) method [6]. Since 1990, the EIA method has been widely used for anti-HCV screening [7]. Butanti-HCV positivity cannot clearly distinguish current, active infection from past infection [8], and positive anti-HCV results can indicate active HCV infection (acute or chronic), past, resolved HCV infection, or a false-positive anti-HCV result [9,10].

However, these tests may give false-positive results in populations where HCV prevalence is low (<3 %). Obtaining positive results close to the cut-off value in anti-HCV tests leads to serious problems for laboratories in routine diagnosis and the need to repeat or confirm tests increases costs [11,12].

In the confirmation of low positive anti-HCV results, the investigation of HCV RNA, which shows viremia, with polymerase chain reaction (PCR) is recommended. In anti-HCV EIA tests, the S/Co value is used as the reactivity threshold, which is obtained

from the ratio of the test sample optic density to the cut-off value, and the manufacturers recommend that S/Co >1 is accepted as positive. Studies conducted in recent years, has reported that the S/Co value is important in the prediction of viremia, high S/Co values are more compatible with HCV RNA, and the most appropriate S/Co values that can be used in the diagnosis of HCV infection are recommended [6,7,12,13].

In this study, a retrospective examination was made of the relationship between anti-HCV and HCV RNA positivity, and it was aimed to determine the most appropriate S/Co value in respect of the performance of the anti-HCV ECLIA test in predicting viremia and the sensitivity and specificity of this test.

Methodology

Patients

From a total of 81, 203 serum samples for health examination tested in the Microbiology Laboratory, between January 2013 and April 2018, the serum samples of 559 patients were positive. In the evaluation of the results, the data of the serum samples in which anti-HCV and HCV RNA were assayed together, were considered.

Anti-HCV assays for HCV infection screening

The anti-HCV tests were assayed with the electrochemiluminescence immunoassay (ECLIA) technique (Elecsys Anti-HCV II assay, Cobas e 601, Roche Diagnostics, Mannheim, Germany). The test results were calculated as the cut-off of signal (S/Co) value obtained from the sample, and according to the manufacturer's recommendation, an S/Co value of <1.0 is accepted as non-reactive and >1 as reactive.

We used ESfEQA (European Society for External Quality Assessment) for proficiency testing four times in a year. We used commercially prepared internal quality control (QC) (positive and negative) two times in a week.

Quantitative RNA PCR

The quantifications of HCV RNA of patients found to be positive were obtained using a commercial viral RNA extraction kit (QIAGEN, QIAsymphony DSP Virus/Pathogen Midi Kit, Hilden, Germany) with the real-time PCR method and on the same day, HCV RNA quantification was studied with a Rotor-Gene RG-Q device (Qiagen, Hilden, Germany) with a PCR kit (artus HCV QS-RGQ Kit, Hilden, Germany). In the study, each run contained 1 negative control, 4 positive control and for all samples we used an internal control. Dynamic range of test was 21 IU/mL. Linear range of test was 21-1×10^8 IU/mL.

Statistical Analysis

By accepting the HCV RNA test as the gold standard determining viremia in the diagnosis of HCV infection, the performance of the anti-HCV test in predicting viremia, and the sensitivity, specificity and predictive values in the data analyses were calculated using SPSS v 15.0 statistics software. To determine the most appropriate anti-HCV reactivity threshold in respect of sensitivity and specificity, receiver operating characteristic (ROC) analysis was applied to evaluate the HCV RNA results of different S/Co values, and the Youden Index was calculated. The Youden Index (*J*), the maximum potential effectiveness of a biomarker, is a common summary measure of the ROC curve [14]. Compatibility between anti-HCV and HCV RNA was evaluated with the Spearman test. Because of the data was not normally disturbed, beside the arithmetic means and standard deviations, median and minimum-maximum values were also used.

Declaration of ethical aspects

Sutcu Imam University Medical Faculty ethics committee approved the study with an issue number 32, dated November 7, 2018.

Results

These 559 patients comprised 302 (54%) males and 257 (46%) females with a mean age of 44.16 ± 20.81 years. In the reactive anti-HCV tests, the median S/Co was 5.78 (min-max, 1.01-265.9). The mean HCV RNA values were determined as $7.8\times10^6 \pm 1.5\times10^6$, median, 2×10^6 (min-max, 720 – 1.5×10^8) copy/mL. When the

Table 1. HCV-RNA negative and positive rates according to the groupings of anti-HCV S/Co values [n (%)].

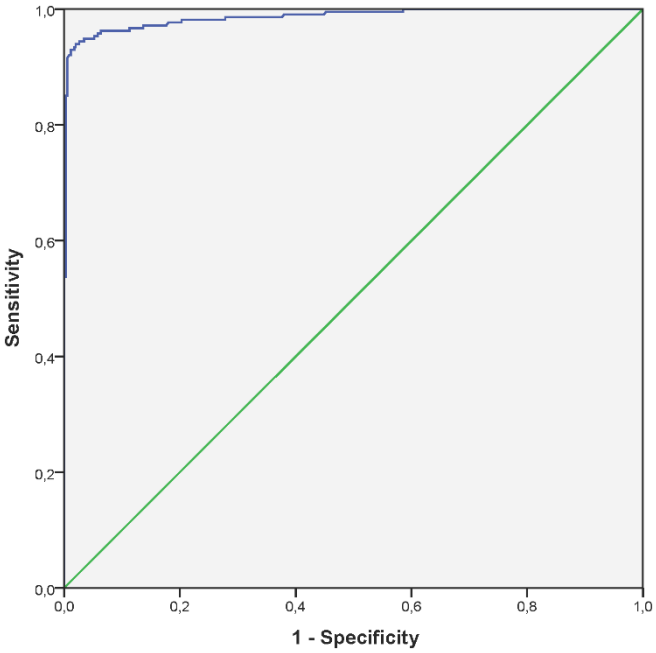
Anti-HCV (S/Co)	HCV RNA NEGATIVE	HCV RNA POSITIVE	TOTAL
1 - 4	234 (67.8%)	3 (1.4%)	237 (42.4 %)
4.1 - 7	62 (18%)	3 (1.4%)	65 (11.6 %)
7.1 - 10	28 (8.1%)	3 (1.4%)	31 (5.5 %)
10.1 - 16	18 (5.2%)	8 (3.7%)	26 (4.7 %)
≥16.1	3 (0.9%)	197 (92.1%)	200 (35.8 %)
TOTAL	345 (100%)	214 (100%)	559 (100%)

S/Co: Signal to cut-off, HCV: Hepatitis C virus.

patients were grouped according to the anti-HCV S/Co values and the HCV RNA results were compared: when S/Co = 1.0-4.0, the positivity rate was (n = 3) 1.4%, S/Co = 4.1-7.0, positivity was (n = 3) 1.4%, S/Co = 7.1-10, positivity was (n = 3) 1.4 %, S/Co = 10.1-16.0, positivity was (n = 8) 3.7% and S/Co ≥ 16.1, positivity was (n = 197) 92.1% (Table 1). Statistically significant correlation was determined between the increase anti-HCV levels S/Co and HCV RNA positivity showing the presence of viremia (p = 0,0001). In the correlation analysis applied between the increase in anti-HCV S/Co values and HCV RNA viral load, no statistically significant correlation was determined (p = 0.244).

In the ROC analysis to be able to determine the best cut-off point, HCV RNA was accepted as the gold standard and accordingly, the most appropriate S/Co value was found to be 12.27 with sensitivity of 94.6%, specifity of 97%, positive predictive value of 96.5% and negative predictive value of 95.7%. In the determination of the cut-off point, the Youden Index values were used (Table 2). The area under the ROC curve (Figure 1) was estimated to be 0.986 (95% confidence interval 0.977-0.995).

Figure 1. Receiver-operating characteristic curve of anti-HCV S/CO ratio for predicting the results of qualitative HCV RNA testing in 559 patients positive for anti-HCV.



HCV, hepatitis C virus; S/CO ratio, signal-to-cutoff ratio; AUC = 0.986 (%95 C.I: 0.977 – 0.995).

Discussion

In the laboratory diagnosis of HCV infection, the algorithm currently applied is generally determination of anti-HCV antibodies as a screening test and in cases of positivity, confirmation with HCV RNA as a more advanced test [6]. HCV RNA is not always consistent with low anti-HCV positivity close to the threshold value. Therefore, HCV RNA methods which are an extremely expensive test, have to be applied in unnecessary cases [15]. Thus, if we could better predict the result of HCV RNA testing, savings in cost and time could be achieved. In the current study, HCV RNA positivity was examined with the real-time PCR method in anti-HCV positive samples, and it was aimed to predict viremia with the determination of the most appropriate S/Co value for the anti-HCV test in the determination of infection.

Table 2. The Sensitivity and Specificity Rates according to HCV RNA of the Anti-HCV S/Co Values of the Samples Selected according to the ROC Analysis.

Anti HCV S/Co	Sensitivity %	Specificity %	PPV	NPV	Youden Index
1.99	100	41,4	51,4	100	0.414
2.97	99,1	58,3	59,6	99,1	0.573
3.97	98,6	67,8	65,5	98,7	0.664
4.98	98,1	75,4	71,2	98,5	0.735
5.94	97,7	80,3	75,5	98,3	0.780
6.92	97,2	85,5	80,6	98	0.827
7.96	96,7	88,4	83,8	97,7	0.851
7.96	96,7	88,4	83,8	97,7	0.851
9.93	95,8	93,9	90,7	97,3	0.897
10.9	94,9	95,9	93,5	96,8	0.908
11.99	94,4	96,5	94,4	96,5	0.909
12.27	**94,4**	**97,4**	**95,7**	**96,6**	**0.918**
15.75	92,1	99,1	98,4	95,3	0.912
18.77	89,7	89,4	84	93,3	0.891
20.09	86	99,4	98,9	92	0.854

PPV, positive predictive value; NPV, negative predictive value; S/CO, signal to cut-off.

Epidemiologically, if the prevalence of a disease is low in a population, then both the false positive rates increase, and the positive predictive value (PPV) of the test in the prediction of actual positivity is decreased. Turkey is one of the countries in the world with a low prevalence of HCV, with a rate of approximately 1% [2]. In accordance with the manufacturer's instructions for the determination of anti-HCV antibodies applied in a routine manner, when the reactivity cut-off point of S/Co = 1 was used, HCV RNA negativity was determined at the rate of 61.7% according to anti-HCV. When the anti-HCV cut-off point of 12.27 was used according to the Youden Index, the false-positive rate was seen to fall to 3.4%. According to our results, the anti-HCV S/CO ratio accurately predicts HCV viremia in patients positive for anti-HCV. At an anti-HCV S/CO ratio cutoff value of 12.27, sensitivity and specificity were high, 94.4% and 97.4%, respectively. Furthermore, these results are consistent with those of several previous studies [7,16,17].

In several published studies, different S/Co values ranging from 3 to 26 were determined in the third generation of anti-HCV assays [18-24]. In a study by Dufour *et al.* the negativity rate was found to be 86% according to HCV RNA in the low positive anti-HCV group and when the cut-off value was taken as S/Co = 3.7, the false positive rate was determined to fall to 10% [18]. In a study of 225 patients, Gurkan *et al.* examined HCV RNA, and determined that all were negative in the range of anti-HCV S/Co value of 1-5, in patients with anti-HCV S/Co value of 5-10, HCV RNA positivity was 16.36% and in those with anti-HCV S/Co value >10, HCV RNA positivity was determined at the rate of 56.22% [12].

In the current study, of the 559 serum samples determined anti-HCV positive with the ECLIA method, 214 (38.3%) were determined with HCV RNA positivity with the real-time PCR method. Statistically significant correlation was determined between the increase anti-HCV levels S/Co and HCV RNA positivity showing the presence of viremia (p = 0,0001). No statistically significant correlation was determined between the increase in the anti-HCV levels (S/Co) and HCV RNA viral load. (p = 0.244), (n = 214).

Sensitivity of 99.7% and specificity of 99.8% have been stated by the manufacturer for the ECLIA technique of the system used in this study (Cobas e 601, Roche Diagnostics, Germany). The manufacturer recommends that an S/Co value of ≥ 1 in this method is accepted as positive. However, several studies have been conducted in respect of raising this value. Ecemis *et al.,* reported that the best sensitivity and specificity rates were obtained when the S/Co was taken as 5 [13]. In a study of 658 patients, Sanlidag *et al.,* could not determine HCV RNA positivity in cases with anti-HCV S/Co < 5.0, and stated that low anti-HCV values shed no light on definitive diagnosis of HCV infection [21]. Some studies have reported that the best S/Co value that increased the sensitivity and specificity was around 10 [16,17,22,23]. In the anti-HCV laboratory tests guidelines of the Centre for Disease Control and Prevention (CDC), it is recommended that when the S/Co value is >8, it is considered positive without the need for any additional test [25]. Fidan *et al.,* studied 297 patients and HCV RNA positivity was not determined in cases with S/Co values <3, and the lowest anti-HCV level determined in HCV RNA positive samples was 10.19 [26].

In the current study, HCV RNA positivity was seen in 7.9% of the patients who had S/Co rate between 1 to 16 and 92.1% of the patients who had S/Co rate above 16. Thus, the HCV RNA positivity rate was determined as 3.4% at anti-HCV values in the range 1-12.27, and as 95.7% at anti-HCV of 12.27 and above. In the ROC analysis applied in the current study to be able to determine the best cut-off value, the most appropriate S/Co value was found to be 12.27, with sensitivity of 94.6%, specificity of 97% and positive and negative predictive values calculated as 97% and 96%, respectively. Unlike previous studies, in the statistical analysis of the current study, when the differentiation threshold value showed a difference in paired classification systems, ROC analysis was applied as the method to be able to determine the best cut-off value. The value of 12.27 was obtained for the S/Co as most consistent with 214 positive HCV RNA tests. According to this value, anti-HCV sensitivity was reduced by 5.6% to 94.4%, and specificity was greatly increased to 97.4%. Moreover, as the most important indicator of the test reliability, PPV was 96.5% and correspondingly, the NPV was very high at 97.5%.

As an example of lower S/Co values, when S/Co = 5.94, sensitivity reached 97.7%, specificity decreased to 80.3%, PPV decreased to 74.5% and NPV increased to 98.2%. In higher S/Co values, despite small increases in specificity, sensitivity was seen to decrease at a greater rate (Table 2). In a study by Seo *et al.* of 661 patients, sensitivity of 73.7% was seen to increase to 94.4% when the S/Co value of 10.9 was used as the most appropriate determined using ROC analysis [22].

In the current study, when anti-HCV S/Co = 1 was used, the sensitivity rate was seen to be at a very good level (100%). However, if the prevalence of disease is low in a population, patient differentiation is as

important as differentiation of healthy individuals. Especially in situations where it is not possible to confirm positive anti-HCV results, the importance of this increases even more. In the current study, the anti-HCV positivity was found to be inconsistent with HCV RNA at the rate of 61.7%, and by taking the value of S/Co = 12.27, this was seen to reduce to 3.4%, and sensitivity was affected at a very low level or, as shown in several studies, did not increase.

In S/Co values < 12.27, sensitivity increased and specificity decreased, a significant decrease from 0.957 to 0.514 was seen in the PPV, which is a marker of the test reliability, and small increases were seen in NPV from 0.966 to 1 (Table 2). When the HCV positivity determined at S/Co values in the range of 1-12 is taken into consideration (12 patients, 2.1% in this study), the most appropriate approach in the evaluation of such results can be said to be the consideration of clinical and biochemical tests together. In an individual with suspicion in the range in question, it may be appropriate to apply the nucleic acid amplification test (NAT), but in an asymptomatic patient, the S/Co value obtained in this range in the anti-HCV test result applied for screening should be treated with suspicion.

Limitation of this study; there are not enough representative samples for the intermediate groups which may confound the positivity rates.

Conclusion

In conclusion, the present study shows that the anti-HCV S/CO ratio is significantly dependent on the presence HCV viremia and that it is highly accurate at predicting the presence of HCV viremia. The results of this study showed that the use of S/Co value of 12.27 as the cut-off value in the anti-HCV test applied with ECLIA was the most appropriate for the determination of HCV infection. For S/Co values below this level, taking the clinical status into consideration before directly examining HCV RNA for confirmation, a more accurate and economical approach could be to run the test again with a new sample (after at least 2 weeks) with the same method in a different device, and /or evaluate together with the clinical findings, or to request an advanced confirmation test and thus it was concluded that HCV RNA should be thought of as the last option. The most appropriate S/Co value should be defined according to the test kit of each laboratory and for the better evaluation of the anti-HCV test, the results report should state the S/Co value and clinicians should be informed on this subject.

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Conflict of interests: No conflict of interests is declared.