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Ortho VITROS enhanced chemiluminescence assay for detection of hepatitis C virus (HCV) antibodies: Determination of a "borderline" range

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The diagnostic efficacy of Ortho VITROS chemiluminescence assay (CIA) in detecting antibodies to the hepatitis C virus (HCV) and the clinical significance of specimens with low sample-to-cut off (S/Co) ratio was analysed, comparing the positive rate for CIA in 5,550 consecutive outpatients with enzyme-immunoassay (EIA). In parallel testing, 43 samples (0.8%) were low positive by CIA (S/Co ratio from 1.0 to 8.0) but negative by EIA. No samples CIA negative/EIA positive were found. Among EIA negative results we found 22 RIBA positive or indeterminate, yielding CIA sensitivities of 100% and EIA sensitivity of 97.8%. None of the 33 samples with CIA S/Co ratios of ≤ 2.0 and only 3 (10.7%) with S/Co ratios of between 2.1 and 8.0 were found to be RIBA positive. Instead, the majority of samples with S/Co ratios ≤ 8.0 (55.7%) were recombinant immunoblotting assay (RIBA) negative. HCV RNA and/ or clinical evidence of HCV infection was not found in any of the 12 indeterminate cases examined with S/Co ratios ≤ 2. We suggest to report them as "Borderline", with the recommendation to follow up in the future.

Key words: HCV infection, RIBA, HCV-RNA, CIA, EIA.

INTRODUCTION

Since their introduction in 1990, enzyme linked immunosorbent assays (EIAs) for antibodies to hepatitis C virus (HCV) have been the principal tests for detection of exposure to HCV. Although usually reported as positive or negative, the results are actually measured as an absorbance signal that is compared with that of a cut-off value (S/C) (Courouce et al., 1991; Zhang et al., 1993; Goncales et al., 2000). It was demonstrated that false-positive anti-HCV results by EIA are frequent in samples with S/C ratio < 3.8 (Dufour et al., 2003). American Centers for Disease Control and Prevention (CDC) recommend RIBA confirmation for these samples(Centers for Disease Control and Prevention, 2003).

It was over ten years ago that a chemiluminescence assay (CIA) for anti-HCV has been developed by Ortho Clinical Diagnostics (Calcagno et al., 2001). The VITROS anti-HCV assay is a specific two-step sandwich CIA for

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the detection of human antibodies to various HCV proteins. Results are usually calculated as a normalized signal-to-cut off (S/Co) ratio. Although several studies have shown that the CIA test is at least as specific and sensitive as conventional enzyme immunoassay (EIA) tests (Griffith et al., 2003; Dufour et al., 2003; Ismail et al., 2004), prompting an increase in its use, it still furnishes a high false-positive ratio, especially in the low-prevalence population. For this reason, further tests such as recombinant immunoblotting assay (RIBA) and/or HCV-RNA PCR are recommended to confirm positive HCV screening results (Richter, 2002; Chevaliez et al., 2006).

In order to provide a systematic approach for the laboratory diagnosis of HCV, in 2003, CDC published guidelines featuring the incorporation of anti-HCV signal-to-cut off (S/Co) ratios into testing algorithms to minimize the number of specimens requiring confirmatory testing. Based on the evaluation of a total of 1,326 reactive samples, supplemental testing was suggested for samples ascribed S/Co ratios of < 8.0 by the VITROS anti-HCV assay (Centers for Disease Control and Prevention, 2003).

The objectives of this study were to evaluate the diagnostic efficacy of the CIA and to assess the relationship between S/Co ratio and RIBA test and HCV RNA, particularly in patients with low CIA ratios (S/Co ≤ 8).

MATERIALS AND METHODS

Sources of samples

The population analyzed in this study comprised 5,550 consecutive outpatients (2,830 females, 51.0% and 2,720 males, 49.0%). The mean age was 47.5 year-old among females (range 18-77 year-old) and 50.5 year-old among male (range 18-83 year-old).

All the patients were recruited between January 2009 and June 2011 from subjects living in the metropolitan area of Naples, Italy and referred to Virology Ambulatory, Transfusion Service, National Tumour Institute “Fondazione G. Pascale” in Naples. All subjects with clinical and biochemical signs of acute hepatitis (such as elevated liver enzyme levels) and those with history of parenteral exposure in the last 6 months were excluded.

No concomitant or autoimmune disorders or underlying systemic disease, including previous malignancy, were included in the present study. Data on risk factors for HCV were unavailable.

Chemiluminescence assays and EIA

All the samples were assessed for the presence of antibodies to HCV using a third generation chemiluminescence assay (CIA) Ortho VITROS anti-HCV (Ortho Clinical Diagnostics) with a sensitivity of 100% and specificity of 99.7%, as indicated by the supplier. Results were calculated as a normalized S/Co value. During the calibration process, a lot-specific parameter, encoded in the lot validation card, was used to determine a valid cut-off value. Samples with S/Co ratio ≥ 1.0 were retested in duplicate and considered “repeatedly positive”.

Repeatedly reactive samples were classified in six groups: the first featured an S/Co of 1.0–2.0, the second an S/Co ranging from 2.1 to 8.0 , the third from 8.1 to 16.0 and then from 16.1 to 20, from 20.1 to 25.0 and the last with S/Co ratios > 25.0.

According to American CDC guidelines, samples with S/C ratio < 8.0 were defined as low positive, while samples with S/Co ratio > 8.0 as high positive (Centers for Disease Control and Prevention, 2003).

All the sera were also tested using a third generation enzyme immunoassay (EIA) Ortho HCV 3.0 (Ortho Clinical Diagnostics) according to the manufacturer’s instructions on an EP100 automated microtiter plate handling system. Although usually reported as positive or negative, the results are actually measured as an absorbance signal that is compared with that of a cut-off value; results above the cut-off are reported as positive, whereas those below the cut-off are called negative. According to American CDC guidelines, samples with S/C ratio < 3.8 were defined as low positive, while samples with S/Co ratio > 3.8 as high positive (Centers for Disease Control and Prevention, 2003).

RIBA testing

The samples positive by CIA were evaluated by RIBA (Ortho Clinical Diagnostics) to confirm the previous results. The testing procedure and assessment of the intensity of the bands were done according to the manufacturer’s instruction. The intensity of the HCV bands was scored in relation to the intensities of the internal IgG controls.

A sample was defined “negative” in absence of any HCV bands, “Indeterminate” if only one band was reactive and “positive” if at least two HCV bands were present.

PCR-RNA

All samples positive by CIA were examined for the presence of HCV RNA with COBAS Ampliprep/Taqman HCV test (Roche Molecular Diagnostics System). Linear range of quantification of the test was 1.50 E+01 to 6.90 E+07 HCV RNA IU/ml, using the accuracy acceptance criterion of +/- 0.3 $\log_{10}$. Specificity of the test was 100% and limit of detection of 15 IU/ml.

RESULTS

The diagnostic efficacy of CIA

Of the 5,550 patients included in this study, 376 (7%) were repeatedly CIA anti-HCV positive and 333 (6%) EIA positive. Comparing the diagnostic efficacy of the CIA and EIA tests, the results were concordant in 5,507 samples (5,174 negative in both the EIA and CIA, 93.6% and 333 positive in both assays, 6.0%) and discordant in 43 samples (0.8%). Of the 333 EIA/CIA positive results, 45 (13.5%) were EIA low positive/CIA high positive, 270 (81.1%) EIA/CIA high positive, while 18 (5.4%) EIA/CIA low positive. There were no samples that were high-positive by CIA that were EIA-negative (Table 1).

The RIBA confirmatory test were performed in discordant samples. Of the 43 that were low positive by CIA (S/Co ratio from 1.0 to 8.0) but negative by EIA, 21

Table 1. Pattern of anti-HCV results in 5,550 samples.

CIA			EIA		
			Negative No. (%)	Positive No. (%)	
				low	high
Negative, No. (%)			5174 (93.6%)	0 (0.0%)	0 (0.0%)
Positive, No. (%)	**Low**		43 (0.8%)	18 (0.3%)	0 (0.0%)
	high		0 (0.0%)	45 (0.8%)	270 (4.5%)

Table 2. RIBA results in relation to S/Co ratios in EIA negative /CIA positive discordant samples.

S/Co ratio	No. of samples	No. (%) found to be RIBA		
		Negative	Indeterminate	Positive
1.0–2.0	33	21 (64.0)	12 (36.0)	0 (0.0)
2.1–8.0	10	0 (0.0)	8 (80.0)	2 (20.0)
Total	**43**	**21(48.8)**	**20 (46.5)**	**2 (4.6)**

(48.8%) were RIBA negative, 20 (46.5%) were RIBA indeterminate, and 2 (4.6%) were RIBA positive (Table 2).

Because RIBA is considered to have virtually 100% specificity for the presence of anti-HCV, we calculated the diagnostic performance of CIA and EIA, using RIBA results as the final determinant of antibody status. Samples that were RIBA negative were considered false positive results, whereas samples that were RIBA indeterminate or positive were considered trues positive for the purpose of the study. Of all CIA negative results, no RIBA positive was found, whereas among CIA positive/EIA negative results we found 2 RIBA positive and 20 RIBA indeterminate (Table 2), yielding sensitivities of 100% and 97.8% respectively. The specificity of the EIA was 98.7% whereas the specificity of the CIA was 96.4%.

The relationship between S/Co ratio and RIBA test and HCV RNA

In order to examine in detail the significance of low-positive results, all samples were classified in six groups in relation to S/Co ratios and tested using RIBA and HCV RNA (Table 3).

None of the 33 samples with S/Co ratios of ≤ 2.0 and only 3 (10.7%) with S/Co ratios of between 2.1 and 8.0 were found to be RIBA positive. Instead, the majority (34/61, 55.7%) of the samples with S/Co ratios of 1.0 to 8.0 were RIBA negative, and 24 (39.3%) were indeterminate. The number of positive RIBAs increased as the S/Co ratio increased, with the highest proportion of RIBA positive samples in the group of samples with S/Co ratios > 25.0 (177/177) (Table 3).

Besides, although the number of samples with detectable HCV RNA increased in relation to the S/Co ratio, the majority of HCV RNA positive samples had an S/Co ratio of > 20 (157/376, 41.7%). In contrast, only 2 of 28 (7.1%) with CIA S/Co ratio between 2.0 and 8.0 were HCV RNA positive. HCV RNA was not detected in any of the samples with CIA S/Co ratios of ≤ 2. These results confirmed that samples with CIA S/Co ratios of ≥ 1 and ≤ 8 have to be classified as low positive, requiring supplemental testing.

To determine whether there was a gradation in likelihood of RIBA positivity in samples with low positive S/Co ratios, we compared the frequency of indeterminate and positive RIBA results and HCV RNA positivity at differing S/Co ratios (Table 4). Among the 61 samples with low-positive CIA results (S/Co ratio ≤ 8.0), 27 (44.3%) were found RIBA positive and indeterminate and only 3 (4.9%) HCV RNA positive, while all the samples (100%) with S/Co ratio > 8.0 were RIBA positive or indeterminate and 163 of 315 (51.7%) HCV RNA positive (Table 4).

The CDC has suggested selecting a S/Co ratio cut-off that identifies 95% of results as RIBA positive and below which 95% of samples are RIBA negative (Albertoni et al., 2010). At a CIA S/Co ratio cut-off of ≤ 8.0, 34 of 34 samples (100%) had negative RIBA results. In contrast, 60 of 60 samples (100%) with S/Co ratio between 8.1 and 20.0 were RIBA positive or indeterminate and 253 of 255 samples (99.2%) with S/Co ratio > 20.0 were RIBA positive (Table 4). No samples that were CIA high positive were RIBA negative.

Among of the samples with CIA S/Co ratios of ≤ 8, 24 were found to be RIBA indeterminate and 3 RIBA positive. These patients were further evaluated for the presence of HCV RNA and for clinical evidence of HCV

Table 3. RIBA and HCV-RNA results for CIA low and high positive samples.

S/Co ratio	No. of samples	No. (%) found to be RIBA			No. (%) found HCV-RNA	
		Negative	Indeterminate	Positive	Negative	Positive
1.0–2.0	33	21 (64.0)	12 (36.0)	0 (0.0)	33 (100)	0 (0.0)
2.1–8.0	28	13 (46.4)	12 (42.9)	3 (10.7)	26 (92.9)	2 (7.1)
8.1–16.0	18	0 (0.0)	11 (61.1)	7 (38.9)	17 (94.4)	1 (5.5)
16.1- 20.0	42	0 (0.0)	2 (4.8)	40 (95.2)	37 (88.1)	5 (11.9)
20.1-25.0	78	0 (0.0)	2 (2.6)	76 (97.4)	42 (53.8)	36 (46.1)
> 25.0	177	0 (0.0)	0 (0.0)	177 (100.0)	56 (31.6)	121 (68.4)
Total	**376**	**34 (9.0)**	**39 (10.4)**	**303 (80.6)**	**211 (56.1)**	**165 (43.9)**

Table 4. RIBA results in relation to S/Co ratios in CIA anti-HCV.

S/Co ratio	No. of samples	No (%) found to be RIBA					
		Negative		Indeterminate		Positive	
		RNA-	RNA+	RNA-	RNA+	RNA-	RNA+
1.0–2.0	33	21	0	12	0	0	0
2.1–8.0	28	13	0	12	0	1	2
8.1–16.0	18	0	0	11	0	6	1
16.1- 20.0	42	0	0	2	0	35	5
20.1-25.0	78	0	0	2	0	42	36
> 25.0	177	0	0	0	0	56	121
Total	**376**	**34(9.0)**	**0(0.0)**	**39(10.4)**	**0(0.0)**	**211(56.1)**	**165(43.9)**

infection by reviewing clinical records. Except for 2/3 RIBA positive, that were found HCV RNA positive with a S/Co ratio between 6.0 and 8.0, all the other samples were found to be HCV RNA negative and with no laboratory evidence of abnormal liver function tests.

DISCUSSION

The increasingly sophisticated methods of diagnosing HCV infection have a direct impact on patient management and the use of more sensitive and specific assays is essential for an efficient diagnosis of HCV infection (Albertoni et al., 2010). Several seroprevalence studies have indicated that S/Co ratios could be used to accurately predict a positive status in conjunction with a confirmatory test (Dufour et al., 2003; Centers for Disease Control and Prevention, 2003; Dufour et al., 2003; Dos Santos et al., 1999; Tobler et al., 2000).

Although the majority of the seroprevalence studies reported were performed using the commercially available EIA test (Centers for Disease Control and Prevention, 2003; Dufour et al., 2003; Dos Santos et al., 1999; Tobler et al., 2000; Albertoni et al., 2010), in the present study we utilized VITROS anti-HCV assay, whose performance was evaluated in some previous published studies. As reported by Ismail et al. (2004), we also found, in the S/Co range of between 1 and 8, EIA negative/CIA positive samples. That clearly shows the highest sensitivity of chemiluminescence than EIA.

On the other hand, our study confirmed the results obtained with EIA in previous studies (Goncales et al., 2000; Dufour, 2004). Low positive samples (EIA S/C ratio < 3.8 and CIA S/Co < 8.0) are commonly false-positive. In fact, the majority of EIA negative/CIA positive samples with S/Co ratios ≤ 8.0 were RIBA negative. Instead, all samples with S/Co ratios > 8.0 were RIBA indeterminate or positive. Our data indicate that EIA has higher specificity than CIA and a reduced sensitivity.

However, despite the apparent true positive nature of these results, HCV RNA was detected only in 6 of 60 (10%) in CIA S/Co ratios of between 8.1 and 20.0. Instead in samples with S/Co ratios of > 20.0, 157 of 255 (61.6%) were HCV RNA positive. There were no such intermediate zones observed with EIA (Dufour et al., 2003). In a study comparing two third-generation EIAs (Goubau et al., 1997), it was found that 98% of samples with high positive anti-HCV by both assays were HCV RNA or RIBA positive. In contrast, samples with discrepant or low positive results were frequently negative on confirmatory tests. With the CIA, the S/Co ratio appears more indicative of HCV RNA status than

was the case for the EIA, were all the results above the cut off values were associated with the same likelihood of obtaining a positive HCV RNA results (Dufour et al., 2003). The reasons for the differences between the EIA and CIA in false positive rates and in correlation between S/Co ratio and HCV RNA are not clear. A difference in antigens used in the two assays cannot explain the difference because a similar discrepancy was showed when comparing EIA with CIA, using the same HCV antigens. Moreover, the CIA is performed in separate reaction cells, making contamination of samples much less likely than EIAs and reducing number of false positive results. By our results, it is clear that the CIA provides several advantages over EIAs, especially in an increased sensitivity, particularly useful in low risk populations, even though all low S/Co ratio samples need use of confirmatory testing. Moreover, the S/Co ratio in CIA positive samples was also predictive of likelihood of HCV RNA positivity.

CDC recommendations suggest that all positive samples with S/Co ratios of ≥ 8.0 can be reported as positive without further supplemental testing (Centers for Disease Control and Prevention, 2003). Although Oethinger et al. (2005) have reported, in samples with CIA S/Co ratios of between 8 and 20, the presence of 12 false positive results (RIBA negative), we do not confirm these data. None of the 315 CIA positive samples with S/Co ratios of ≥ 8.0 were RIBA negative. Moreover, we showed that the majority (61.6%) of 255 samples with CIA S/Co ratios of >20 were HCV RNA positive. Watterson et al. (2007) reported that only 1 sample (4%) of Vitros low positive samples with S/Co ratios of < 10 were RIBA positive, while Dufour et al. (2003) found that 13 of 129 (10%) samples with S/Co ratios of < 8.0 were RIBA positive. In contrast, we found that 3 of 61 (4.9%) of samples with an S/Co ratio of ≤ 8.0 were RIBA positive. These data seem to confirm what was observed by other authors that reported that only 6 of 203 (3%) of samples with an S/Co ratio of < 8.0 were RIBA positive (Oethinger et al., 2005). However, they stated that all RIBA negative samples in that range had an S/Co ratios of < 5.0. This finding seems to be confirmed by Contreras et al. (2008) that shows 4.5 to be the optimal cut-off point for the S/Co ratio to identify the majority (95%) of Vitros anti-HCV false positive results. In our study, we found that all RIBA negative samples had an S/CO ratios of ≤ 2.0. The reason for the difference is uncertain but could be due to the difference in sample size or population examined.

Our study has several strengths: the samples size was sufficiently large and supplemental testing, both 3rd – generation RIBA and HCV RNA, were performed on all samples. However, some limitations of the study should be considered. First of all, we did not determine the specific causes of false-positive anti-HCV results and our proposal is only applicable when the 3rd-generation Ortho Vitros anti-HCV assay is used. Evaluation of other currently available assays is warranted to define the optimal of antibodies that can be used to identify false-positive results with the objective of eliminating unnecessary supplemental testing.

Conclusion

In this study, we failed to find any evidence of HCV infection in any sample with a S/Co ratio of ≤ 2; no sample considered was found to be RIBA positive, a majority of samples was RIBA negative and none of the 12 RIBA indeterminate cases examined were eventually found to be HCV-RNA positive. So we have recently opted to report all our CIA low positive samples with S/Co ratios between 1 and 2 as “Borderline”, with the recommendation that follow-up testing should be performed when HCV infection continues to be suspected based on other clinical or laboratory information, as recently documented (Zer et al., 2009; Lai et al., 2011; Seo et al, 2009)

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