

# Indeterminate RIBA results were associated with the absence of hepatitis C virus RNA (HCV-RNA) in blood donors

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## ABSTRACT

**Introduction:** Hepatitis C virus (HCV) infection is diagnosed by the presence of antibodies and is supplemented by confirmatory testing methods, such as recombinant immunoblot assay (RIBA) and HCV-RNA detection. This study aimed to evaluate the efficacy of RIBA testing to diagnose HCV infection in blood donors positive for anti-HCV antibodies. **Methods:** A total of 102 subjects positive for anti-HCV determined by enzyme-linked immunosorbent assay (ELISA) at the Hematology and Hemotherapy Foundation of Bahia (HEMOBA) were later assessed with new samples using the Abbott Architect anti-HCV test (Abbott Diagnostics, Wiesbaden, Germany), the RIBA III test (Chiron RIBA HCV 3.0 SIA, Chiron Corp., Emeryville, CA, USA), the polymerase chain reaction (PCR; COBAS® AMPLICOR HCV Roche Diagnostics Corp., Indianapolis, IN, USA) and line probe assay (LiPA - Siemens, Tarrytown, NY, USA) genotyping for HCV diagnosis. **Results:** Of these new samples, 38.2% (39/102) were positive, 57.8% (59/102) were negative and 3.9% (4/102) were indeterminate for anti-HCV; HCV-RNA was detected in 22.5% (23/102) of the samples. RIBA results were positive in 58.1% (25/43), negative in 9.3% (4/43) and indeterminate in 32.6% (14/43) of the samples. The prevailing genotypes were 1 (78.3%, 18/23), 3 (17.4%, 4/23) and 2 (4.3%, 1/23). All 14 samples with indeterminate RIBA results had undetectable viral loads (detection limit  $\leq 50$  IU/mL). Of these samples, 71.4% (10/14) were reevaluated six months later. Eighty percent (8/10) of these samples remained indeterminate by RIBA, and 20% (2/10) were negative. **Conclusions:** In this study, individuals with indeterminate RIBA results had no detectable HCV-RNA.

**Keywords:** Blood donors. Hepatitis C virus. Anti-HCV. RIBA. HCV-RNA.

## INTRODUCTION

The hepatitis C virus (HCV) is a worldwide public health concern, with an estimated 2.2% of the world population suffering from infection. This prevalence translates into roughly 130 million HCV carriers, with chronic infections occurring in approximately 70% of all cases<sup>1</sup>. A population-based national survey of viral hepatitis conducted in Brazilian capitals measured the HCV prevalence to be between 0.9% and 1.9%<sup>2</sup>. In a population-based study conducted in 1998 in Salvador, the capital of the northeastern State of Bahia, the HCV prevalence was 1.5%<sup>3</sup>.

The laboratory diagnosis of HCV infection is generally performed with serological testing for anti-HCV antibodies or using molecular biological methods for HCV-RNA detection.

Currently, alternative enzyme-linked immunosorbent assay (ELISA) methods, such as chemiluminescence assays (CLIAs) or microparticle enzyme immunoassays (MEIAs), which employ the same antigens as ELISAs, can be used to detect anti-HCV antibodies<sup>4</sup>. CLIA offers a significantly improved specificity and positive predictive value while maintaining a sensitivity that is comparable to ELISA<sup>5</sup>. False-positive results for anti-HCV are likely in populations with a low HCV prevalence, such as blood donors, or in instances when cross-reactivity occurs due to the presence of other viral antigens or antibodies in individuals with immune disorders. False-negative results may occur in immunosuppressed populations, such as HIV-infected patients, solid organ transplant recipients, hypoglobulinemia or agammaglobulinemia patients or hemodialysis patients<sup>6,7</sup>.

The CDC (Centers for Disease Control and Prevention, Atlanta, GA, USA) recommends that all anti-HCV screening be confirmed using supplementary serological testing or NAT (nucleic acid testing). RIBA (recombinant immunoblot assay) is the preferred supplementary serological testing method due to its robust specificity. RIBA testing requires the identification of false-positive results using ELISA, particularly when considering populations with low HCV prevalence rates, such as blood donors, students and general populations lacking known risk factors<sup>8</sup>. RIBA detects the reactivity of antibodies

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to antigens that are immobilized on nitrocellulose immunoblot strips. When no reactions are observed against any of the evaluated antigens, a RIBA test is considered negative. When a reaction is registered against only one protein, it is then considered indeterminate. However, when reactivity is observed against two or more proteins, the result is considered positive<sup>9</sup>.

Indeterminate RIBA results have been observed in recently infected individuals who are undergoing the seroconversion process and occasionally in populations that are chronically infected with HCV. Indeterminate outcomes may also result from false positives during screening, most commonly in populations at low risk for HCV infection<sup>10</sup>. The present study observed that indeterminate RIBA results were significantly associated with the absence of HCV-RNA, suggesting that this test may be unnecessary, particularly in low-risk populations, such as blood donors.

## METHODS

### Study population

A total of 62,123 samples from blood donors were screened at the Hematology and Hemotherapy Foundation of Bahia (HEMOBA) between June 1, 2009 and December 22, 2010 for

anti-HCV antibodies using ELISA and a HEPANOSTIKA<sup>®</sup> HCV Ultra assay kit with a DAVINCI<sup>®</sup> system microElisa instrument (Beijing United Biomedical Co., LTD.). Of these samples, 0.48% (298/62,123) tested positive, while 99.52% (61,825/62,123) tested negative. We collected new samples from 34.2% (102/298) of the individuals who tested positive for anti-HCV by ELISA at the Hematology and Hemotherapy Foundation of Bahia (HEMOBA) for confirmatory HCV diagnosis. The protocols used in this study were approved by the Ethics and Research Institutional Review Board of the Oswaldo Cruz Foundation (FIOCRUZ-Bahia), case number 193/2009, protocol 294. All included patients underwent an interview, answered a questionnaire and provided written informed consent. **Figure 1** depicts an illustration of the overall study design.

### Hepatitis C virus antibody detection and confirmatory RIBA testing

All study participants were reassessed for the presence of anti-HCV antibodies at the Central Laboratory of Public Health of Bahia (LACEN-BA) using the ARCHITECT Anti-HCV CLIA (Abbott Diagnostics, Wiesbaden, Germany) in an Architect i4000 (Abbott Diagnostics) automated immunology analyzer. All serological testing was performed and analyzed

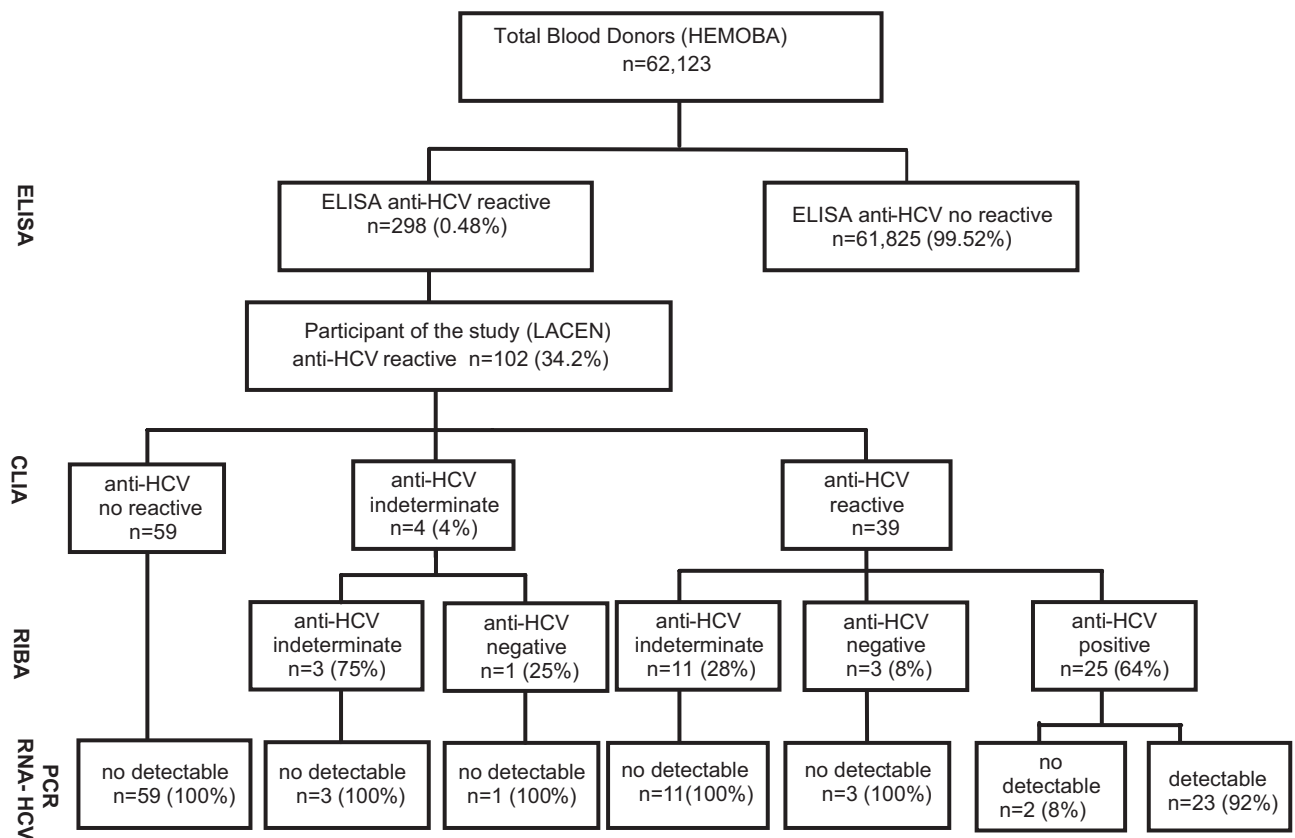


FIGURE 1 - Flowchart: Prevalence of anti-HCV blood donors: June 1, 2009 – December 22, 2010. ELISA: enzyme linked immuno sorbent assay; CLIA: chemiluminescence assays; RIBA: recombinant immunoblot assay. PCR: polymerase chain reaction; RNA-HCV: ribonucleic acid-hepatitis C virus; HEMOBA: Hemotherapy Foundation of Bahia. LACEN: *Laboratório Central de Saúde Pública*.

in accordance with manufacturer guidelines. The samples were considered positive for anti-HCV antibodies when the index values (S/CO) were  $>1.1$ , non-reactive when values were  $<0.9$  and indeterminate when values ranged between 0.9 and 1.1.

The samples with positive and indeterminate results for anti-HCV were further assessed for anti-HCV antibodies using a RIBA 3.0 Strip Immunoblot Assay (Chiron RIBA HCV 3.0 SIA, Chiron Corp., Emeryville, CA, USA).

### Molecular analysis

The qualitative detection of HCV-RNA was performed using the AMPLICOR® Hepatitis C Virus (HCV) Test v2.0 (Roche Diagnostics Corp., Indianapolis, IN, USA) with a detection limit of 50 IU/mL. Genotyping was performed using the VERSANT HCV Genotype 2.0 Assay (LiPA) (Siemens, Tarrytown, NY, USA) with a reverse hybridization method. HCV-RNA was quantified with either the AMPLICOR HCV MONITOR® Test v2.0 (Roche Diagnostics Corp., Indianapolis, IN, USA) using real-time PCR or the COBAS® AmpliPrep/COBAS® TaqMan® HCV Test (Roche Diagnostics Corp., Indianapolis, IN, USA), which also utilizes RT-PCR. All tests were performed and analyzed in accordance with manufacturer guidelines.

### Statistical analysis

All data obtained in this study were entered into the Epi Info™ database v3.5.1, which is maintained by the Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA). Statistical data analysis was performed using Epi Info v3.5.1. Results were considered statistically significant when the p-value  $< 0.05$ .

## RESULTS

A total of 102 blood samples were used to compare RIBA test results with those from standard HCV tests. The mean age of the blood donors was  $37.9 \pm 10.1$  years, and 56.9% (58/102) of the donors were male. New blood samples were collected from each individual and submitted for retesting at LACEN-BA to detect anti-HCV using the ARCHITECT anti-HCV assay (Abbott Diagnostics, Wiesbaden, Germany). Of these samples,

38.2% (39/102) were positive, 4% (4/102) were indeterminate and 57.8% (59/102) were negative for anti-HCV antibodies. Anti-HCV index values  $\geq 5$  were most the common values (61.5%, 24/39) observed. **Table 1** delineates the classifications of all of the anti-HCV index values measured in this study.

RIBA testing was performed on 43 samples, including 39 positive and 4 indeterminate for anti-HCV antibodies using CLIA. Of the samples, 58.1% (25/43) were positive, 32.6% (14/43) were indeterminate, and 9.3% (4/43) were negative. Of the positive RIBA samples, 60% (15/25) presented reactivity for all (4) antigens present on the nitrocellulose immunoblot test strips (**Table 2**). The observed reactivities for c100, c33, c22 and NS5 antigens in these RIBA-positive samples were 84%, 100%, 96% and 68%, respectively (**Table 3**).

Of the 14 samples that were indeterminate by RIBA, reactivity was observed in the c33 (86%; 12/14) and c22 (14%; 2/14) bands, with the following band intensity levels: 1+ (50%; 7/14), 2+ (14%; 2/14) or 3+ (36%; 5/14). No reactivity was detected in the c100 and NS5 bands in these samples.

RIBA was repeated six months later on some of the individuals who received an indeterminate initial RIBA result (71.4%; 10/14). Of these individuals, only two received negative results, while all of the other results remained indeterminate. Similar band patterns were observed in these samples, and 50% (4/8) of the samples were considered weak for band intensities (1+ or 2+).

HCV-RNA was successfully detected using qualitative PCR in 92% (23/25) of the RIBA-positive samples. Of the two positive RIBA samples that had no detectable HCV-RNA, one sample presented reactivity in bands c100, c22 and c33, while the other presented reactivity in bands c33 and NS5. The order of genotype prevalence was as follows: genotype 1 (78.3%; 18/23), genotype 3 (17.4%; 4/23) and genotype 2 (4.3%; 1/23) (**Table 3**). HCV-RNA was undetectable in all of the samples with indeterminate or negative RIBA results, and the HCV viral load was quantified in each sample with detectable HCV-RNA, as shown in **Table 1**.

Of the samples with viral loads  $>850,000$  IU/mL (56.5%; 13/23), eight samples presented reactivity to the four antigens (c100, c33, c22 and NS5) evaluated using RIBA, while four

TABLE 1 - Comparison of RIBA test results, anti-HCV index values and HCV-RNA.

RIBA	Anti-HCV index value										Total
	$\geq 1$		$\geq 2$		$\geq 3$		$\geq 4$		$\geq 5$		
	n	%	n	%	n	%	n	%	n	%	
Negative	2	25.0	1	20.0	1	25.0	0	0.0	0	0.0	4
Indeterminate	6	75.0	4	80.0	2	50.0	2	100.0	0	0.0	14
Positive	0	0.0	0	0.0	1	25.0	0	0.0	24	100.0	25
HCV-RNA (%)	0	0.0	0	0.0	0	0.0	0	0.0	23	96.0	23
Total	8	100.0	5	100.0	4	100.0	2	100.0	24	100.0	43

HCV-RNA: Hepatitis C virus-ribonucleic acid; RIBA: recombinant immunoblot assay.

TABLE 2 - Frequency of positive bands observed in RIBA testing.

Number of positive RIBA bands	Absolute frequency (n)	Relative frequency (%)
1*	14	36.0
2	3	8.0
3	7	18.0
4	15	38.0
Total	39	100.0

\*RIBA indeterminate. RIBA: recombinant immunoblot assay.

samples had reactivity in three out of four bands and one sample reacted in just two of the bands. In the samples with viral loads <850,000 IU/mL (43.5% 10/23), seven had reactivity in all four bands, two presented reactivity in three bands and one presented reactivity in just two bands. The lowest viral load detected in this study was 30,200 IU/mL, with reactivity observed in bands c33 and c22 using RIBA; accordingly, the highest viral load observed was 69,000,000 IU/mL, with reactivity evidenced against all four antigens. The number of bands present on the immunoblot strips and the observed reactivity patterns using RIBA were subsequently correlated with the presence of HCV-RNA and genotype, as shown in **Table 3**.

TABLE 3 - Comparison of detected antigens, presence of viremia and HCV genotypes in RIBA-positive samples.

Number of bands	RIBA test antigens				Total (%)	HCV-RNA (%)	HCV genotype				
	c100	c33	c22	NS5			1	2	3		
4	+	+	+	+	15	60.0	15	65.0	13	0	2
3	+	+	+	-	6	24.0	5	22.0	4	0	1
	-	+	+	+	1	4.0	1	4.0	0	1	0
2	-	+	+	-	2	8.0	2	9.0	1	0	1
	-	+	-	+	1	4.0	0	0.0	0	0	0
Total (n)	21	25	24	17	25		23		18	1	4
(%)	84.0	100.0	96.0	68.0	100.0		100.0		78.3	4.3	17.4

HCV-RNA: Hepatitis C virus-ribonucleic acid; RIBA: recombinant immunoblot assay

## DISCUSSION

The presence of anti-HCV antibodies was confirmed in only 38.2% (39/102) of 102 individuals with positive anti-HCV results using CLIA at LACEN-BA. The discrepancy between the results obtained at HEMOBA and the results from this study may be largely explained by the use of different serological diagnostic testing techniques for HCV detection. Blood banks generally employ extremely sensitive testing methods to avoid the risk of HCV transmission via transfusion, while public health laboratories use tests that offer improved specificity.

In low-risk populations, such as blood donors, HCV screening lacks specificity, as approximately 60% of reagent samples return false-positive results<sup>11</sup>. These false-positive anti-HCV results may be the product of cross-reactivity with immunoglobulins that are present in populations of African descent or in individuals with myeloma, rheumatoid factor, liver diseases (such as cirrhosis and cancer), autoimmune diseases (collagenous, autoimmune hepatitis) or other viral infections (such as HIV or hepatitis B). False positives may also result when serum samples are stored for extended periods, when serum samples are subjected to temperature variations or when an individual has received prior immunization<sup>12</sup>.

In this study, HCV was detected in 53.5% (23/43) of the participants who tested positive or indeterminate for anti-HCV antibodies. This detection level represented a lower percentage than that of a study conducted in blood donors from Midwestern Brazil, which reported HCV detection in 80.5% (165/205) of subjects who were positive for anti-HCV (using ELISA)<sup>13</sup>. In 92% (23/25) of the RIBA-positive samples in which HCV-RNA was detected and the corresponding anti-HCV index value was >5.0, a statistically significant association was observed between elevated anti-HCV index values and the presence of HCV-RNA (p-value <0.01). Indeterminate results were observed in 32.6% (14/43) of the samples submitted to RIBA, which is in contrast with the lower percentage of indeterminate RIBA samples that was previously reported<sup>14</sup>.

In samples that were considered positive or indeterminate (18.6%, 19/102) and that had an anti-HCV index value (S/CO) <5.0 (i.e., those indicating low positivity), 73.7% (14/19) had RIBA-indeterminate results. In addition, eleven samples presented antibodies that bound to antigen c33, while three samples had antibodies that bound to c22. HCV-RNA was not detectable in any of these samples, which is consistent with results that have been previously reported<sup>8,11,15,16</sup>. Nonetheless, in other studies<sup>17</sup>, the virus was successfully detected in some indeterminate RIBA samples.

One plausible explanation for the low positivity observed herein with respect to anti-HCV antibodies and negative or indeterminate RIBA results with no detected viremia could involve instances in which past HCV infections occurred or were eliminated without total elimination of the antibodies<sup>11,18</sup>. In this regard, several strategies have been employed to clarify the significance of indeterminate RIBA results in the absence of HCV-RNA, including risk factor analysis, alanine aminotransferase levels, anti-HCV index values, previous blood donations and reactivity intensity (detected in a single band using confirmatory immunoblot assay testing)<sup>17,19</sup>.

Of the 71.4% (10/14) of samples that initially received an indeterminate RIBA result and that were submitted to retesting six months later, eight had similar results, with identical band patterns and undetectable HCV-RNA, while two had negative results using subsequent confirmatory RIBA testing. These findings are consistent with data that have been presented previously<sup>17,20</sup>.

The present study found no correlation between viral load and the number of positive bands observed using RIBA analysis, despite the HCV viral load being high in 65% (15/23) of the samples that showed reactivity in all four bands. In the samples demonstrating reactivity in two bands (c33 and c22), viral load was detected in just two. It was not possible to confirm any relationship between the presence of viremia and the number of bands with observed reactivity using RIBA due to the insufficient sample size employed in this study and the low frequency of viremia detected in positive RIBA samples with reactivity in two bands, which indicated the elimination of HCV infection<sup>21</sup>.

The most prevalent genotype observed in this study was genotype 1, followed by genotype 3, which is in agreement with previous findings<sup>22,23</sup>. The presence of all four bands in the genotype 1 samples may be explained by the increased prevalence of this genotype in the present study and the fact that the proteins considered by the RIBA nitrocellulose test strips pertain to this genotype.

Indeterminate RIBA results were reported in 13.7% (14/102) of the samples obtained from the individuals included in this study, indicating the need for molecular testing as a confirmatory measure in the diagnosis of HCV infection. All of these samples had anti-HCV index values <5.0 and undetectable HCV-RNA, and 64% (9/14) of the samples showed weak band intensity (1+ or 2+). However, individuals who received positive RIBA results with the presence of viremia had anti-HCV index values >5.0 and strong band intensity (3+ or 4+). In conclusion, in individuals with indeterminate RIBA test results, it is highly likely that HCV-RNA will be undetectable when the detection limit is 50IU/mL.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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