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Study on Reliability of Commercially Available Hepatitis C Virus Antibody Tests

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The serodiagnosis of hepatitis C virus (HCV) infection was analyzed by a recombinant immunoblot assay (RIBA) with recombinant proteins encoded by the viral RNA isolated from our patients in Hamburg, Germany. The HCV RNA was amplified by PCR, and proteins encoded by the viral core and the NS3, NS4, and NS5 regions were expressed subsequently in *Escherichia coli*. The results obtained with our UKE RIBA were compared with the results of the Abbott HCV second-generation enzyme immunoassay (EIA). Serum samples from 270 patients, which were sent to us on the suspicion of HCV hepatitis and which were negative for hepatitis A virus and hepatitis B virus antibodies, were examined. In 227 cases (84.1%), there were identical positive (204 cases, 75.6%) or negative (23 cases, 8.5%) results in both tests. In 32 cases (11.9%), the reactive Abbott second-generation HCV EIA results could not be confirmed by the UKE RIBA and the HCV PCR. In follow-up studies conducted over 1 year, these results did not change. In three cases (1.1%), the UKE RIBA presented a positive result while the Abbott second-generation HCV EIA was negative. All three cases were positive in the HCV PCR and showed seroconversion in an HCV EIA 4 to 6 weeks later. In addition, 33 patient serum samples were examined by UKE RIBA in parallel with the Ortho RIBA 2.0. In three cases (9.1%), a positive Ortho RIBA 2.0 result could not be confirmed by the UKE RIBA and the HCV PCR. All three patients were free of complaints. The UKE RIBA showed also a smaller number of indeterminate results (3.0%) than the Ortho RIBA 2.0 (24.2%). This comparison study demonstrates that the commercially available HCV antibody tests should be further improved.

The hepatitis C virus (HCV) has been identified as the most frequent causative agent of parenterally transmitted non-A, non-B hepatitis (5).

First-generation HCV enzyme immunoassays (EIA) detected only antibodies against nonstructural region 4 (NS4) with recombinant antigen c100-3 (11). The tests of the second generation, the Abbott HCV EIA and the Ortho recombinant immunoblot assay (RIBA) 2.0, used additional antigens of the core region (c22-3), the NS3 region (c33c), and (especially the Ortho RIBA 2.0) a part of c100-3 (named 5-1-1) from the NS4 region.

In daily diagnostic work, the commercially available Abbott second-generation HCV EIA and the supplementary Ortho RIBA 2.0 often do not yield clear indications of whether an HCV infection exists.

For that reason, we developed our own serological test system and examined whether it would provide more reliable results than the commercially available tests.

MATERIALS AND METHODS

PCR. PCR was performed as previously described (16). Briefly, RNA was extracted by the guanidium thiocyanate-phenol method (4). After reverse transcription, amplification was done in a buffer of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 160 μM (each) deoxynucleoside triphosphate, 30 pmol of each sense or antisense primer, and 2 U of Ampli*taq* (Perkin-Elmer Cetus, Emeryville, Calif.). For routine diagnosis, we used a primer of the 5′ nontranslated region (Table 1). Amplification products were separated by electrophoresis in 2% 3:1 NuSieve agarose (FMC, Rockland, Maine), blotted onto positively charged nylon membrane (Qiagen, Hilden, Germany), and hybridized with a radioactively labeled probe.

For the recombinant proteins, we used primers of the core, NS3, NS4, and NS5 regions of the HCV genome (Table 1).

Cloning and expression of recombinant proteins. The primers for the different regions of HCV were synthesized with *Eco*RI (5′) and *Hin*dIII (3′) restriction sites. Amplification products were cloned in the pBluescript II vector (Stratagene, La Jolla, Calif.) and were sequenced by the chain-terminating method (17). For expression, we used the vector pGEX-2T (Pharmacia, Freiburg, Germany) for recombinant proteins c1, NS4, and NS5b and the vector pMAL (New England Biolabs, Beverly, Mass.) for proteins c2, NS3, and NS5a. After transformation in *Escherichia coli* DH5α (Gibco BRL, Gaithersburg, Md.), expression was induced by addition of IPTG (isopropyl-β-D-galactopyranoside) with a final concentration of 0.3 mM for 2 h at 37°C. Subsequently, 50-ml aliquots of the bacterial suspensions were put on ice for 15 min and spun down at 1,600 × *g* at 4°C. The bacterial pellets were resuspended in 6 ml of an ice-cold solution of 20 mM Tris-HCl (pH 7.4), 200 mM NaCl, and 1 mM EDTA and were frozen overnight at −20°C. After slowly thawing on ice, the bacterial suspensions were sonified at 100 W in eight cycles of 20 s on ice. The nonsoluble part of the sonified solution was spun down at 12,000 × *g* for 5 min, and the supernatant was collected. The HCV fusion proteins were separated from *E. coli* proteins by affinity chromatography. For the pGEX system, we used glutathione 4-B Sepharose (Pharmacia), and for the pMAL system, we used maltose-binding protein–agarose (New England Biolabs) as recommended by the manufacturer.

Immunoblots. The six different soluble HCV fusion proteins were fixed on a polyvinylidene difluoride (PVDF) membrane (Millipore, Eschborn, Germany) by the convertible filtration manifold slot blot system (Gibco BRL). Briefly, 1 μg of the HCV fusion protein was suspended in 185 μl of 20 mM Tris-HCl (pH 7.4)–200 mM NaCl. Each fusion protein solution was transferred into a separate slot and blotted onto a PVDF membrane. As an internal control, 1-μg and 375-ng quantities of immunoglobulin G (IgG) from an HCV-negative standard serum (Behring, Marburg, Germany) and 1-μg quantities of glutathione *S*-transferase and maltose-binding protein were fixed on a PVDF membrane. After that, the PVDF membrane was blocked with a 5% solution of skim milk (Oxoid, Basingstoke, England) by agitation on a rotation shaker for 1 h. Then, the membrane was cut into 25 strips vertical to the slots of the blot apparatus.

For immunoblot assay, serum samples were diluted 1:100 in 20 mM Tris-HCl (pH 7.4)–150 mM NaCl–0.05% Tween 20 (TBST) and were exposed to blot strips for 1 h on a rotation shaker at room temperature. Then, the strips were washed three times with 4 ml of TBST for 5 min. After that, the strips were exposed to alkaline phosphatase-labeled goat anti-human-IgG antibodies (Sigma, Deisenhofen, Germany) diluted 1:7,500 in TBST for 30 min on a rotation shaker. Again, the strips were washed three times with 4 ml of TBST for 5 min and finally were washed with 4 ml of TBS (TBST without Tween 20). The strips were developed with a substrate solution (Western Blue; Promega, Heidelberg, Germany) of BCIP (5-bromo-4-chloro-3-indolylphosphate toluidinium) and Nitro Blue Tetrazolium.

Our RIBA was considered positive when antibodies against at least two pro-

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TABLE 1. Sequences of primers used in this study

Region and primer[a]	Sequence	Nucleotide positions[b]
Core		
c1 (s)	TCAGTTGAATCCAAACGTAACACCAA	371–384
c2 (as)	GTACTGAAGCTTAGGCCGGGAGC	655–645
c3 (c-DNA; as)	GTCAAGCTTCACGCCGTCCTCCAGA	827–812
NS3		
3lo (s)	CTGTTGTGCCCCGCGGGGCAC	3846–3866
3lo (c-DNA; as)	AAGCCCCGGGGTGTTCATGTA	4985–4965
3l (s)	TCAGGAATTCGTGGCTAAGGCGGTGGACTT	3906–3925
3l (as)	GTCAAGCTTAAGCTTCTAACAGCCTGCGTCATAGCACTC	4919–4896
NS4		
4lo (s)	ATTGCTTCATTGATGGCTT	5697–5715
4lo (c-DNA; as)	GAGGCTGCTGAGTATGGCAGT	6191–6171
4li (s)	TCAGAATTCTCCTGTTCAACAT	5760–5772
4li (as)	GTCAAGCTTAAACGTGGTTACCC	6129–6116
NS5		
n1 (s)	CATGAATTCTGCGTCCAACC	8028–8038
d95 (c-DNA; as)	CGTAAGCTTAGTCATAGCCTCCGTGAA	8630–8613
n5 (s)	ACTGAATTCTCGTATGATACCCGC	8249–8263
n2 (as)	GTCAAGCTTAGCCGCAGTTCT	8432–8421
5′ untranslated region		
11 (s)	TTCGCGGCCGCACTCCACCATGAATCACTCCCC	1–17
12 (as)	AGTCTTGCGGCCGCAGCGCCAAATC	255–244
13 (c-DNA; as)	CCCAACACTCGCCTA	276–265

[a] s, sense; as, antisense; c-DNA, primer for reverse transcription.
[b] Numbered according to the sequence published by Choo et al. (6).

teins of different regions of HCV and no antibodies against the control proteins glutathione *S*-transferase and maltose-binding protein could be detected.

If only antibodies against a protein of a single region of HCV could be detected, the result of the immunoblot assay was indeterminate, similar to those of the commercial assays.

The commercial tests, the Abbott second-generation HCV EIA and the Ortho RIBA 2.0, were performed as recommended by the manufacturers.

Nucleotide sequence accession numbers. Sequences of the HCV genome have been assigned the accession numbers X78950 to X78955 at the EMBL data library.

RESULTS

Immunoblots. The RNA isolated from the sera of the HCV-infected patients was amplified with primers for different regions of HCV: core, NS3, NS4, and NS5. The amplification products were cloned in plasmid pBluescript II (Stratagene) before being sequenced. Our HCV sequences showed homologies of 79.5 to 91.4% for nucleotides and 88.0 to 94.7% for amino acids to the prototype HCV-1 (6).

After subcloning in the vectors pMAL (c1, NS4, and NS5a) and pGEX (c2, NS3, and NS5b), six soluble fusion proteins were expressed in *E. coli* and isolated from bacterial proteins by affinity chromatography (Fig. 1).

The isolated soluble fusion proteins were fixed on a PVDF membrane and the immunoblot assay was performed as described in Materials and Methods. Using different immunoblot batches, we always obtained identical results.

To control the specificity of our UKE RIBA, we tested serum samples from 100 patients whose cases were familiar to us in repeated HCV diagnostic tests for at least 2 years. All these samples were negative in hepatitis A and hepatitis B virus antibody detection tests. The sera of 50 patients with repeated positive results in HCV second-generation EIA (optical density [OD], >2.2) and HCV PCR for at least 2 years who also exhibited clinical signs of liver disease (elevated levels of liver enzymes and histopathologically confirmed hepatitis) showed all positive results in UKE RIBA. The negative control group (50 patients) were all asymptomatic for liver disease and tested negative in HCV second-generation EIA and HCV

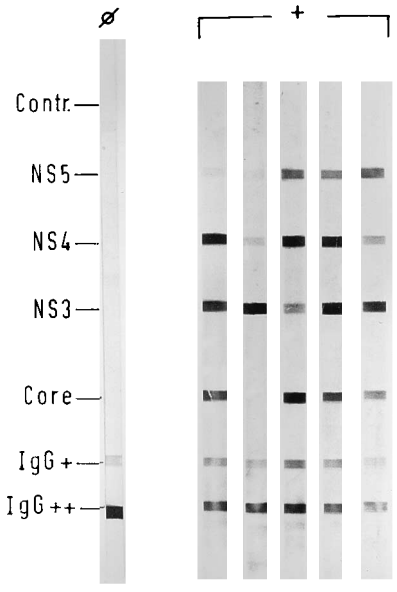

FIG. 1. UKE RIBA results for six different serum samples: one negative (Ø) and five positive (+). To each strip, 40 ng (IgG++) or 15 ng (IgG+) of IgG and 40 ng of the native expression protein (Contr.) were applied. For detection of specific antibodies against HCV, 40 ng of each of the recombinant proteins of four different HCV regions (core, NS3, NS4, and NS5 regions) was used. The five positive serum samples contained antibodies against proteins of at least two different regions.

TABLE 2. Comparison of Abbott second-generation EIA with UKE RIBA

UKE RIBA result (n = 270)	HCV EIA result	
	No. (%) of positive samples	No. (%) of negative samples
Positive	204 (75.6)	3 (1.1)
Negative	32 (11.9)	23 (8.5)
Indeterminate	3 (1.1)	5 (1.8)

PCR at the time of the investigation. Serum from all 50 patients stayed negative in repeated HCV tests (EIA and PCR) 3 to 6 months after UKE RIBA investigation. For all these 50 sera, the UKE RIBA showed a negative result.

Antibodies against the proteins glutathione *S*-transferase and maltose-binding protein were not detectable.

Comparison of the UKE RIBA with the Abbott second-generation EIA. To compare the results of our UKE RIBA with those of the second-generation HCV EIA, we took 270 serum samples which were sent to our laboratory on suspicion of first-time HCV infection. All were tested again 3 to 6 months later (Table 2).

Two hundred four patients (75.6%) had positive results in both tests which stayed positive for several months. All of these patients had clinical signs of chronic liver disease starting 6 months to 25 years earlier. Sixty-six patients (32.4%) had known risk factors for acquiring HCV infection. Forty-two patients (20.6%) had experienced polytransfusion, 19 patients (9.3%) had histories of intravenous drug abuse, and 5 patients (2.5%) underwent chronic hemodialysis for several years.

In 23 cases (8.5%), both tests, the Abbott second-generation HCV EIA and the UKE RIBA, showed negative results and remained negative in tests repeated months later.

In 32 cases (11.9%), the positive Abbott second-generation EIA results could not be confirmed by the UKE RIBA. Furthermore, the HCV PCR showed a negative result for all 32 sera. The extinction ODs of the Abbott second-generation HCV EIA were in the range of 0.5 to 1.5, with a mean OD of 0.98. In follow-up studies carried out over 1 year, there was no significant increase of the extinction values in the Abbott second-generation EIA. The results of the UKE RIBA and the HCV PCR remained negative. All 32 patients were free of complaints, and no patient had elevated levels of transaminases. The 32 patients were not immunosuppressed (e.g., by transplantation, human immunodeficiency virus infection, or chronic hemodialysis), and no one had a history of blood transfusion or of any other known risk factor.

In three cases (1.1%), the UKE RIBA showed a positive result while the Abbott second-generation HCV EIA was negative. All three sera showed antibodies against the proteins of the core and NS3 regions of HCV in the UKE RIBA and were positive by HCV PCR. The three patients had elevated levels of transaminases since a few weeks earlier. After this first investigation, their sera tested positive in the Abbott second-generation EIA 4 to 6 weeks later. Two of the three patients had histories of intravenous drug abuse, and one patient was hemodialyzed for several years.

There were indeterminate UKE RIBA results in eight cases (2.9%). Five sera showed reactivity against the protein of only the NS5 region of HCV in the UKE RIBA and were negative in the second-generation EIA and the HCV PCR. In three cases (1.1%), reactivity against the protein of only the viral NS3 region was detectable in the UKE RIBA. The second-generation EIA and the HCV PCR presented positive results in all three cases.

TABLE 3. Agreement of UKE RIBA-positive or -indeterminate results with PCR results

UKE RIBA protein reactivity[a]					PCR-positive samples[b]
Core	NS3	NS4	NS5	Total no. of samples	
+	+	+	−	60	24/24
+	+	+	+	59	19/19
−	+	+	−	26	7/7
+	+	−	+	24	6/6
−	+	+	+	13	7/7
+	+	−	−	12	3/3
+	−	+	+	8	5/5
+	−	+	−	5	3/5
−	+	−	−	3	3/3
−	−	−	+	5	0/5

[a] The totals of protein samples reacting positively or indeterminately in UKE RIBAs were as follows: 168 core samples, 197 NS3 samples, 173 NS4 samples, 109 NS5 samples, and 215 samples in total.

[b] Number of PCR-positive samples/number of UKE RIBA-positive samples. In total, results for 77 of 84 tests were in agreement.

For the 207 cases in which the UKE RIBA showed positive results, there was a difference in the frequencies of reactivity against the recombinant proteins (Table 3). In 194 of 207 cases (93.7%), the reactivity against the protein of the NS3 region was the most frequent, followed by reactivities of the proteins of the NS4 (82.6%) and core (81.2%) regions. Antibodies against the recombinant protein of the NS5 region were found in only 50.2% of the samples. In eight cases, the UKE RIBA presented an indeterminate result. Of these cases, results for only the three sera which were reactive for the NS3 region could be confirmed by HCV PCR. For the five sera which were reactive against the recombinant protein of only the NS5 region, no positive HCV PCR result could be detected. Sera from these five patients remained negative in follow-up tests, and the patients were asymptomatic during the clinical course. The patients were not immunosuppressed.

Comparison of UKE RIBA with Ortho RIBA 2.0. To compare the Ortho RIBA 2.0 with the UKE RIBA, we investigated 33 serum samples, of which 25 were positive and 8 were indeterminate in the Ortho RIBA 2.0 (Table 4). All samples were positive in the second-generation HCV EIA at the time of the investigation. In 19 cases (57.5%), the UKE RIBA showed an

TABLE 4. Comparison of test results for 33 sera by UKE RIBA and Ortho RIBA 2.0

No. of cases (n = 33)	Results of test[a]								
	UKE RIBA for protein				PCR	Ortho RIBA 2.0 for protein			
	Core	NS3	NS4	NS5		c22-3	c33c	c100-3	5-1-1
14	+	+	−	+/−	+	+	+	−	−
5	+	+	+	−	+	+	+	+	+
3	+	+	−	−	+	+	+	+	−
3	−	−	−	−	−	+	+	+	−
4	−	−	−	−	−	−	−	+	−
1	−	+	+	−	+	−	+	−	−
1	+	+	−	−	+	+	−	−	−
1	+	+	+	+	+	−	+	−	−
1	−	+	−	−	−	−	+	−	−

[a] +, positive; −, negative; +/−, indeterminate.

identical positive result, with reactivity against viral proteins from the core, NS3, and NS4 regions. In three cases (9.1%), there were antibodies against the core and NS3 regions detectable in both assays but the Ortho RIBA 2.0 presented in addition reactivity against the viral protein of the NS4 region (c100-3). These 22 sera positive in the UKE RIBA and the Ortho RIBA 2.0 were also positive by HCV PCR.

The sera of three patients, which were positive in the Ortho RIBA 2.0, were found to be negative in the UKE RIBA. In all three cases, the HCV PCR was also negative. The Abbott second-generation HCV EIA presented an extinction value that was less than 0.8 in all cases. The serum of one of the three patients became negative in the second-generation HCV EIA in follow-up studies 3 months later but showed an identical positive result in the Ortho RIBA 2.0. All three patients were free of complaints, had no disease which could lead to immunosuppression, and did not show elevated levels of transaminases. In follow-up studies carried out over 1 year, serum from each of the three patients remained negative in the HCV PCR and the extinction values of the second-generation HCV EIA did not increase. The patients stayed asymptomatic of clinical signs for hepatitis.

From the eight sera (24.2%) that showed indeterminate results in the Ortho RIBA 2.0, only one (3.0%) presented an indeterminate result in the UKE RIBA too. In three cases, the results of the reactive UKE RIBA were confirmed by the HCV PCR and by clinical signs of chronic hepatitis. In four cases, negative results were found in both the UKE RIBA and the HCV PCR. These four patients were asymptomatic and had no immunosuppression from transplantation, human immunodeficiency virus infection, or hemodialysis.

DISCUSSION

It is well known that the commercial second-generation HCV EIA presents false reactive results (7, 8, 19). Likewise, the commercial Ortho RIBA 2.0 as a supplementary test, which uses the same antigens as the second-generation EIA for antibody detection, often presents indeterminate and reactive results that cannot be confirmed by PCR (2, 12).

One of the major problems in daily routine work is to decide whether a patient is HCV infected. In a number of cases, the commercial tests did not help us to decide whether an HCV infection existed. What is the significance of second-generation EIA extinction ODs of less than 1.5? What is the significance of an indeterminate Ortho RIBA 2.0 result? Is the patient infected or not? We receive many serum samples which show extinction ODs of less than 1.5 in the second-generation HCV EIA or from patients with clinical signs of hepatitis in whose sera we cannot detect antibodies against an infectious agent. The detection of HCV RNA by PCR leads to definite results. But until now this method has been too laborious for the screening of hundreds of serum samples. If the PCR results are negative, it is difficult to differentiate between the degradation of viral RNA during transport, low levels of viremia, and the status of virus elimination. For this reason, we developed our own UKE RIBA with recombinant antigens expressed in *E. coli* after amplification of HCV from the sera of our patients.

In comparing UKE RIBA and Abbott second-generation EIA, there are identical positive or negative results in 227 of 270 tests (84.1%) (Table 3). In 32 cases (11.9%), the Abbott second-generation EIA showed a false reactive result whereas the UKE RIBA and the HCV PCR presented negative results in agreement with the clinical course. All 32 cases yielded extinction values below 1.5 in the EIA, and there was no increase in follow-up studies conducted over 1 year. This result suggests that the Abbott second-generation EIA results were false reactive.

In three cases (1.1%), the UKE RIBA detected antibodies against the core and NS3 regions of HCV earlier than the second-generation HCV EIA, which uses recombinant proteins from the same regions, did. There was a diagnostic gap of 4 to 6 weeks in the Abbott second-generation EIA compared with the UKE-RIBA.

The results of the Ortho RIBA 2.0 revealed a lack of specificity in comparison with the UKE RIBA, the HCV PCR, and the clinical course of patients in follow-up studies (Table 4). The status of one of the three patients whose sera were positive in the Ortho RIBA 2.0 and negative in the UKE RIBA as well as in the HCV PCR could not be confirmed by the Abbott second-generation EIA, in spite of the fact that this test contains proteins identical to those in the Ortho RIBA 2.0. Furthermore, the Ortho RIBA 2.0 showed indeterminate results in 24.2% of our collective samples. This high rate of indeterminate results agrees with rates for other investigations (3, 14).

Our results show that in the Ortho RIBA 2.0, the c100-3 protein in particular is responsible for this false-positive reactivity (Table 4). This recombinant protein, which was used in the first-generation EIAs, is known for its unspecific reactivity (10, 19, 20). The Ortho RIBA 2.0 does not fulfill all of the demands of a confirmatory test for HCV diagnosis. The Ortho RIBA 2.0 uses antigens identical to those of the second-generation EIA and carries over the lack of specificity and sensitivity of this test.

In contrast to the Ortho RIBA 2.0, we used different recombinant proteins for the UKE RIBA. Our UKE RIBA detects the HCV infection earlier than the Abbott second-generation EIA does.

The more reliable results of the UKE RIBA in comparison with those of commercial antibody detection tests may in part be due to the facts that we used local HCVs to generate the recombinant protein and that the serological response tested was from local specimens. The UKE RIBA is also able to detect antibodies of patients who are infected with HCV genotypes 1, 3, and 4 per a recently published classification (18). In 107 isolates from our patients, only these three different genotypes were found by sequencing a part of the NS5 region of HCV. Although the number of serum samples from foreign patients examined with the UKE RIBA is small, the results in these cases suggest that the UKE RIBA reliability is not limited to the patients from Hamburg.

The frequency of detection of antibodies against recombinant proteins by the UKE RIBA reveals that the NS3 region plays a particularly important role in HCV diagnosis (Table 3). This region codes for a protease and a helicase (1, 9, 13). We detected antibodies against the NS3 region in 93.7% of the 207 UKE RIBA-positive serum samples, and we found a strong antibody reaction against the NS3 region particularly early in HCV infection. On the other hand, we found antibodies against the NS5 region in only 50.2% of the samples. This region codes for an RNA-dependent RNA polymerase and exhibits cross-reactivity with other viruses (15). All five sera which showed reactivity with only the antigens derived from the NS5 region were negative for HCV infection in the PCR, and the patients were asymptomatic in the clinical course. Therefore, we consider this antigen of no use for diagnostic purposes.

Until now, the absence of a "gold standard" for HCV antibody tests has made it difficult to compare the sensitivities and specificities of diagnostic assays. The lack of detectable specific antibodies against HCV does not exclude the possibility of an infection, especially during the acute phase of infection or for

patients who are immunosuppressed because of transplantation, cancer, human immunodeficiency virus infection, or chronic hemodialysis, for example. On the other hand, PCR provides only a measure of viremia and the absence of HCV RNA by PCR does not exclude the possibility of an HCV infection in all cases. It is possible that in different phases of the HCV infection viremia is absent or below the level of detection. Besides this problem, early separation of serum and cells and appropriate transport of the samples are very important to prevent false-negative PCR results. The diagnostic work is further complicated by the fact that the presence of normal transaminases does not exclude the possibility of an HCV infection. But when the commercial assay is reactive (especially with an OD of less than 1.5) and the UKE RIBA is negative, an unspecific reaction can be assumed if the patient is without elevated levels of transaminases and HCV PCR is negative. A worldwide standard for HCV antibody testing should be established. Our results show that the commercially available HCV antibody tests need to be improved.

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