JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 2001, p. 1665–1668 Vol. 39, No. 4
0095-1137/01/$04.00+0 DOI: 10.1128/JCM.39.4.1665–1668.2001

Relevance of Reactivity in Commercially Available Hepatitis C Virus Antibody Assays

SUSANNE POLYWKA,* MATTHIAS SCHRÖTER, HEINZ-HUBERT FEUCHT, BERNHARD ZÖLLNER, AND RAINER LAUFS

Institute for Medical Microbiology and Immunology, University Hospital Eppendorf, 20246 Hamburg, Germany

Received 7 September 2000/Returned for modification 21 November 2000/Accepted 23 January 2001

Sera from 2,148 patients were tested with a third-generation microparticle enzyme immunoassay (MEIA), a confirmatory assay, and a reverse transcription-PCR. Overall, 85.6% of reactivities were confirmed, 13.2% were shown to be unspecifically reactive, and 1.2% were indeterminate. The rate of confirmed MEIA reactivities clearly depended on the strength of the reactivity.

Earlier, a high rate of false-positive enzyme immunoassay (EIA) results measured by a commercially available second-generation hepatitis C virus (HCV) EIA, as compared to an in-house confirmatory assay (Universitäts-Krankenhaus Eppendorf strip immunoblot assay [UKE SIA]), was described (14). To assess whether inclusion of the NS5 protein in screening assays of the third generation enhanced sensitivity and specificity, we compared the reactivity measured by a third-generation microparticle EIA (MEIA) to that measured by our confirmatory assay and reverse transcription-PCR (RT-PCR). Additionally, we wanted to assess whether breakpoints could be defined to differ between reactivities which can usually be confirmed and those which are most often not specific.

(Parts of this study were presented at the 39th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, Calif. [abstr. H-87].)

Included in this study were patients whose sera were tested for HCV antibodies by MEIA and immunoblotting and for viral RNA by RT-PCR. Between April 1998 and March 1999, sera drawn from 2,148 patients with suspected HCV infection were included in the study. Patients' ages varied from 1 to 90 years (mean, 40.9 years). From 1,587 patients, additional serum samples were available which had been taken before or after the study. In the case of discrepant results between MEIA, immunoblotting, and/or HCV PCR, test results obtained on other occasions were used to classify patients as infected or not infected.

The AxSYM MEIA (HCV version 3.0; Abbott GmbH Diagnostika, Wiesbaden-Delkenheim, Germany) used as a screening assay contains four recombinant proteins: HCr43, a fusion protein consisting of parts of the structural core region and the NS3 region; c200, containing parts of the NS3 and NS4 regions; c100-3, containing a shorter sequence of the NS3 region and the same part of the NS4 region; and NS5, using parts of the NS5 region (HCV version 3.0, manufacturers' instructions; Abbott GmbH Diagnostika). Tests were conducted according to the manufacturers' instructions. Sera were considered reactive if their optical value (S/CO) was greater than or equal to the cutoff value (S/CO $\geq$ 1.0). In the case of an S/CO value between 0.8 and 0.99, the serum was classified as borderline reactive and the MEIA was repeated.

As a confirmatory assay we used the previously described (6, 14) UKE SIA, which used four recombinant proteins derived from the NS5, NS4, NS3, and core regions of HCV which were different from those used in commercially available tests (6). As described earlier, UKE SIA was considered positive if there were at least two positive bands and at least one band showed an intermediate or high reactivity (6). If a reactivity against only two proteins with an intensity of less than intermediate or against only one antigen was observed, the result was rated indeterminate. PCR was performed as described previously (12) using primers of the 5′ nontranslated region. In October 1998 we changed the amplification technique by using the LightCycler (Roche-Boehringer Mannheim, Mannheim, Germany) (16). The lower detection limit of both techniques is 10^2 copies/ml. Genotypes were determined serologically or by nucleotide sequencing of the NS5 region as described previously (15).

Of 2,148 patients tested, 101 were negative by MEIA, 16 had borderline reactivity, and 2,031 were positive. Ninety-seven of the 101 patients who had negative MEIA results were also negative by UKE SIA and PCR, confirming that they were not infected with HCV. In two cases, UKE SIA was also negative but PCR showed a clearly positive result (1×10^6 copies/ml and 2×10^7 copies/ml). One of the patients was a 61-year-old female from Russia with myelodysplastic syndrome. She had not received blood products recently and had no other risk factors for the acquisition of HCV, but she had histologically confirmed liver cirrhosis. She was not infected with the hepatitis B virus and had no markers of autoimmune hepatitis or primary biliary cirrhosis. Her HCV infection was confirmed in a second serum sample drawn 2 weeks later when she still had no antibodies to HCV, as tested by MEIA or UKE SIA, but PCR was clearly positive (5×10^5 copies/ml). The HCV genotype was 1b. The other patient was a 25-year-old male medical student who started intravenous drug use in September 1998. In December 1998, both antibody assays were negative but PCR revealed 10^7 copies/ml. In a second serum sample drawn 12 days later, antibody reactivity was absent in the

* Corresponding author. Mailing Address: Institute for Medical Microbiology and Immunology, University Hospital Eppendorf, Martinistraße 52, 20246 Hamburg, Germany. Phone: 49 40 42803 3147. Fax: 49 40 42803 4881. E-mail: polywka@uke.uni-hamburg.de.

TABLE 1. PCR and UKE SIA results for MEIA-reactive sera

MEIA result (S/CO)	*n*	No. (%) of patients with indicated UKE SIA result					
		PCR-positive sera			PCR-negative sera		
		Positive	Indeterminate	Negative	Positive	Indeterminate	Negative
1–10	311	12 (3.9)	4 (1.3)	1 (0.3)	17 (5.5)	21 (6.8)[a]	256 (82.3)[b]
11–20	89	19 (21.3)	5 (5.6)		46 (51.7)	5 (5.6)[c]	14 (15.7)[d]
21–30	85	34 (40.0)	4 (4.7)	1 (1.2)	43 (50.6)	2 (2.4)	1 (1.2)
31–40	100	53 (53.0)	3 (3.0)	1 (1.0)	41 (41.0)	2 (2.0)[e]	
41–50	110	59 (53.6)	4 (3.6)		44 (40.0)	2 (1.8)[f]	1 (0.9)
51–60	153	94 (61.4)	4 (2.6)		54 (35.3)		1 (0.7)
61–70	208	133 (63.9)	1 (0.5)		73 (35.1)	1 (0.5)	
71–80	249	182 (73.1)			66 (26.5)	1 (0.4)	
81–90	281	193 (68.7)			88 (31.3)		
91–100	241	173 (71.8)	1 (0.4)		66 (27.4)	1 (0.4)[g]	
>100	204	146 (71.6)			58 (28.4)		
Total	2,031	1,098 (54.1)	26 (1.3)	3 (0.1)	596 (29.3)	35 (1.7)	273 (13.4)

[a] Three of these patients had a positive PCR result earlier (two of them were treated with IFN) and were considered infected. One other patient was only transiently positive by MEIA but negative in all tests 5 months earlier and 2 months later and was thus considered not infected.
[b] Four of these patients had positive PCR and antibody results earlier and lost these during recovery; one of them was coinfected with HIV-1.
[c] Two of these patients were considered infected. One of them was tested during treatment with IFN, and one had strong antibody reactivities earlier.
[d] Two of these patients were considered infected. One of them had positive PCR results before IFN treatment, and one had an acute infection and became highly reactive in the antibody assays later.
[e] One of these patients was proven to be infected due to highly positive PCR results prior to treatment with IFN.
[f] Both patients were HCV infected. One was a child with perinatally acquired HCV infection with earlier positive PCR results, and the other was a drug-addicted female patient with earlier positive PCR results.
[g] This patient was HCV infected; he had highly positive PCR results prior to treatment with IFN.

screening assay and UKE SIA but PCR was positive with 2×10^7 copies/ml. Four weeks later, UKE SIA was still negative but MEIA was weakly reactive, with an S/CO ratio of 3.9, and PCR was positive (10^6 copies/ml), confirming an acute HCV infection. The genotype was 3a.

Two additional MEIA-negative serum samples were positive in the confirmatory assay, but we could not detect HCV RNA by RT-PCR. No further serum samples were available in these cases to confirm the reactivity in UKE SIA.

None of the 16 patients with borderline reactivities was positive by RT-PCR or had any band in UKE SIA; therefore, we found no evidence of ongoing HCV infection.

Overall, 2,031 serum samples were reactive for HCV antibodies in the screening assay. The distribution of S/CO values is shown in Table 1. Positive MEIA results were confirmed in 1,723 cases: in 29 PCR was positive, in 596 UKE SIA was positive, and in 1,098 both PCR and UKE SIA were positive. For 1,428 of these patients, further serum samples were obtained over a period of 1 month to 16 years; HCV infection was confirmed in all of them.

For 35 patients, PCR was negative and the blot revealed an indeterminate result, so a classification of "infected" or "not infected" based on this serum alone could not be made. Nine of these patients had tested positive by PCR or UKE SIA in a serum sample drawn earlier (0.5 to 7 years) and were classified as infected. Four of these patients became PCR negative with a decline in antibody reactivity as shown by UKE SIA during therapy with interferon (IFN), which might have led to a decrease in antibody response. One of them was also positive for human immunodeficiency virus type 1 (HIV-1), which may have contributed to the weak antibody reactivity, and one patient was treated shortly after she acquired HCV. Another patient was a 7-year-old girl with perinatally acquired HCV infection who tested positive for viral RNA during her first year of life and whose serum remained reactive with HCV antibodies throughout follow-up but was only weakly reactive by UKE SIA. In two other cases, HCV infection was confirmed by a positive UKE SIA result in another serum sample drawn 4 months to 4 years earlier. The remaining two patients had confirmed HCV infection as shown by positive PCR results 2 and 4 years earlier. One additional patient had only a transiently positive antibody assay, reacted solely with the core antigen of the UKE SIA, and due to negative results in all tests 5 months earlier and 2 months later was classified as not infected. For 25 patients a further classification could not be made since we did not obtain further samples ($n = 10$) or their results remained indeterminate during follow-up for 4 months to 4 years (median, 11 months) ($n = 15$).

The MEIA reactivities of the remaining 273 sera (13.4%) were not confirmed by PCR or blotting. In four of these cases, the S/CO values were low, between 1.6 and 5.4. In sera drawn 1, 3, 4, and 5 years earlier, EIA reactivities were confirmed by both positive UKE SIA and positive PCR results; three of these patients were drug addicts. The decrease in antibody reactivity and the subsequently negative PCR suggest that the patients were HCV infected but cleared the infection, leaving a weakly reactive MEIA result. However, spontaneous loss of HCV is a very rare event (7) and thus probably does not explain the majority of low reactivities. Two further samples with S/CO values of 12 and 17 were derived from drug-addicted patients; one had been positive by blotting and PCR 5 years earlier, before he was started on IFN. The other was an HIV-1-infected female who had acute HCV infection with negative results by all assays 3 months earlier. She became PCR positive 14 months later, confirming ongoing HCV infection, but due to her immunosuppressed condition she showed no reactivity by UKE SIA.

Thus, 1,738 positive MEIA results (85.6%) were found in

TABLE 2. Final classifications of MEIA-reactive sera

MEIA result (S/CO)	*n*	No. (%) of sera with indicated result		
		True positive	Indeterminate	True negative
1–10	311	41 (13.2)	17 (5.5)	253 (81.4)
11–20	89	74 (83.1)	3 (3.4)	12 (13.5)
21–30	85	82 (96.5)	2 (2.4)	1 (1.2)
31–40	100	99 (99.0)	1 (1.0)	
41–50	110	109 (99.1)		1 (0.9)
51–60	153	152 (99.3)		1 (0.7)
61–70	208	207 (99.5)	1 (0.5)	
71–80	249	248 (99.6)	1 (0.4)	
81–90	281	281 (100.0)		
91–100	241	241 (100.0)		
>100	204	204 (100.0)		
Total	2,031	1,738 (85.6)	25 (1.2)	268 (13.2)

patients with ongoing or former HCV infection (Table 2). A total of 268 sera (13.2%) could be classified as falsely reactive, which is unacceptably high. For 107 of these patients we had follow-up sera over a period of 1 month to 8 years (median, 9 months); 16 were only transiently reactive by MEIA, and the others always had positive MEIA results which could not be confirmed by another test. The incidence of this false reactivity clearly depended on the S/CO value. While 81.4% of reactivities with an S/CO value of less than 10 were shown to be false, only 0.9% of those between 41 and 50 could not be confirmed by PCR or blotting, and a false-positive result was not detected in any sera with S/CO values of more than 60 (Table 2).

In 25 cases (1.2%) a final classification was not possible. The number of indeterminate test results (i.e., a single band in the confirmatory assay and a negative PCR) also varied with the S/CO values. Only 5.5% of sera with S/CO values of less than 10 could not be classified, and this number declined with stronger MEIA reactivities. However, even with S/CO values of more than 61 there were two patients for whom a final classification was not possible. These patients are especially hard to advise. Immunological disorders may contribute to a low or absent antibody response, as has been described for patients on chronic hemodialysis (2, 13). However, we have no evidence that underlying immunosuppression was the cause of a weak immune response in those 35 patients with indeterminate test results. Single bands in confirmatory assays using recombinant proteins or unconfirmed low MEIA reactivities may be caused by antibodies against the microorganism used for cloning and expression (10). Thus, synthetic peptides have been described to be more specific, but unfortunately they have proven to be less sensitive (3, 10). High levels of immunoglobulin G may lead to nonspecific binding of the Fc fragment to the solid phase, resulting in false reactivity in a screening assay. Therefore, high rates of unconfirmed reactivities can be found in patients with rheumatoid arthritis, Sjögren's disease, autoimmune hepatitis, primary biliary cirrhosis, or mixed cryoglobulinemia (8).

The inclusion of an antigen from the NS5 region in third-generation HCV screening assays led to enhanced sensitivity, but this was achieved by modification of the c33 protein (4, 9). Our study shows that these screening assays have a propensity for false reactivities. In blood donors for whom the positive predictive value of a positive HCV screening assay is described to be low (1), an unspecific reactivity would lead to rejection of an otherwise healthy blood donor. But in patients with clinical signs of ongoing hepatitis, weak antibody reactivity without confirmation might lead to an incorrect diagnosis of HCV infection and unjustified treatment, which is both expensive and marked with side effects.

In conclusion, diagnosis of HCV infection should never be based on a positive result in a screening assay alone. To achieve a more reliable diagnosis, sera with positive results in an EIA should always be retested by a confirmatory assay based on immunodominant antigens clearly different from those used in screening assays to exclude both assays from detecting the same false reactivity (11). Second, HCV diagnosis is hampered by the restriction of antigens used in commercially available tests to genotype 1a, which is predominantly found in the United States; these antigens do not necessarily represent viral genotypes found in other parts of the world (3, 5, 8). Thus, our confirmatory assay based on local isolates proved to be superior to commercial assays (6) and led to a very low number of patients for whom a final diagnosis was not possible (1.7%). Patients with indeterminate results and immunosuppressed patients should always be tested by PCR. Thus, PCR not only is necessary to estimate the infectivity of an infected patient but often serves as a confirmatory assay. However, low-level viremia is found in chronically infected patients; a negative PCR result in a serum sample does not exclude ongoing HCV infection nor does it prove spontaneous recovery from HCV (7). A laboratory should carefully evaluate the screening assay and establish cutoff values below which a positive result can most often be considered unspecific. In MEIA, this breakpoint is 10, while S/CO values of more than 30 can normally be confirmed.

REFERENCES

1. **Bresters, D., H. L. Zaaijer, H. T. M. Cuypers, H. W. Reesink, I. N. Winkel, P. J. van Exel-Oehlers, A. A. J. van Drimmelen, P. L. M. Jansen, C. L. van der Poel, and P. N. Lelie.** 1993. Recombinant immunoblot assay reaction patterns and hepatitis C virus RNA in blood donors and non-A, non-B hepatitis patients. Transfusion **33:**634–638.
2. **Bukh, J., P. Wantzin, K. Krogsgaard, F. Knudsen, R. H. Purcell, R. H. Miller, and the Copenhagen Dialysis HCV Study Group.** 1993. High prevalence of hepatitis C virus (HCV) RNA in dialysis patients: failure of commercially available tests to identify a significant number of patients with HCV infection. J. Infect. Dis. **168:**1343–1348.
3. **Callahan, J. D., N. T. Constantine, P. Kataaha, X. Zhang, K. C. Hyams, and J. Bansal.** 1993. Second generation hepatitis C virus assays: performance when testing African sera. J. Med. Virol. **41:**35–38.
4. **Damen, M., H. L. Zaaijer, H. T. M. Cuypers, H. Vrielink, C. L. van der Poel, H. W. Reesink, and P. N. Lelie.** 1995. Reliability of the third-generation recombinant immunoblot assay for hepatitis C virus. Transfusion **35:**745–749.
5. **Dhaliwal, S. K., L. E. Prescott, B. C. Dow, F. Davidson, H. Brown, P. L. Yap, E. A. C. Follett, and P. Simmonds.** 1996. Influence of viraemia and genotype upon serological reactivity in screening assays for antibody to hepatitis C virus. J. Med. Virol. **48:**184–190.
6. **Feucht, H.-H., B. Zöllner, S. Polywka, and R. Laufs.** 1995. Study on reliability of commercially available hepatitis C virus antibody tests. J. Clin. Microbiol. **33:**620–624.
7. **Feucht, H. H., B. Zöllner, M. Schröter, S. Polywka, P. Buggisch, H. Nolte, and R. Laufs.** 1999. High rate of chronicity in HCV infection determined by antibody confirmatory assays and PCR in 4110 patients during long-term follow-up. J. Clin. Virol. **13:**43–51.
8. **Gross, J. B., and D. H. Persing.** 1995. Hepatitis C: advances in diagnosis. Mayo Clin. Proc. **70:**296–297.
9. **Lee, S. R., C. L. Wood, M. J. Lane, B. Francis, C. Gust, C. M. Higgs, M. J. Nelles, A. Polito, R. DiNello, and D. Achord.** 1995. Increased detection of hepatitis C virus infection in commercial plasma donors by a third-generation screening assay. Transfusion **35:**845–849.
10. **Léon, P., J. A. López, and J. M. Echevarría.** 1993. Evaluation of laboratory assays for screening antibody to hepatitis C virus. Transfusion **33:**268–270.

11. **Nübling, C. M., G. von Wangenheim, S. Staszewski, and J. Löwer.** 1994. Hepatitis C virus antibody among immunodeficiency virus-1-infected individuals: analysis with different test systems. J. Med. Virol. **44:**49–53.
12. **Reuter, D., S. Polywka, L. Iske, H. H. Feucht, and R. Laufs.** 1992. Close correlation between hepatitis C virus serology and polymerase chain reaction in chronically infected patients. Infection **20:**320–323.
13. **Schröter, M., H. H. Feucht, P. Schäfer, B. Zöllner, and R. Laufs.** 1997. High percentage of seronegative HCV infections in hemodialysis patients: the need for PCR. Intervirology **40:**277–278.
14. **Schröter, M., H.-H. Feucht, P. Schäfer, B. Zöllner, S. Polywka, and R. Laufs.** 1999. Definition of false-positive reactions in screening for hepatitis C virus antibodies. J. Clin. Microbiol. **37:**233–234.
15. **Schröter, M., H.-H. Feucht, P. Schäfer, B. Zöllner, and R. Laufs.** 1999. Serological determination of hepatitis C virus subtypes 1a, 1b, 2a, 2b, 3a, and 4a by a recombinant immunoblot assay. J. Clin. Microbiol. **37:**2576–2580.
16. **Schröter, M., B. Zöllner, P. Schafer, R. Laufs, and H.-H. Feucht.** 2001. Quantitative detection of hepatitis C virus RNA by LightCycler PCR and comparison with two different PCR assays. J. Clin. Microbiol. **39:**765–768.