

Evaluation of Commercial Enzyme Immunoassays for Detection of Hepatitis Delta Antigen and Anti-Hepatitis Delta Virus (HDV) and Immunoglobulin M Anti-HDV Antibodies

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Panels of hepatitis B virus surface antigen-positive sera from drug abusers were used to evaluate 14 commercial enzyme immunoassays from six companies for detecting hepatitis delta virus (HDV) markers. For detecting hepatitis delta virus antigen (HDAg), the Wellcome, Pasteur and Noctech assays had 100% sensitivity for all 42 HDAg-positive serum specimens that were confirmed in-house; the Organon reagents gave 59.5% sensitivity without detergent and 64.3% sensitivity with detergent, but there were 14 discrepant results with and without detergent. The Sorin assay detected HDAg in only 10 of the positive samples (23.8% sensitivity). For the detection of antibody to HDV (anti-HDV) all six commercial enzyme immunoassays were reactive with all 36 anti-HDV-positive specimens that were confirmed in-house. There were no false-positive results with the Wellcome, Noctech, or Sorin assay, but one specimen was false positive by the Organon assay. One HDAg-positive specimen gave a false anti-HDV-positive result in the Abbott assay and an equivocal result in the Pasteur assay (97.8% specificity). For the detection of immunoglobulin M anti-HDV, the Wellcome, Noctech, and Sorin assays agreed for the 38 positives confirmed in-house, except for one false negative with the Sorin test. We conclude that there has been a substantial improvement over previously evaluated assays in sensitivity and specificity of commercial assays for anti-HDV detection, and the sensitivities of immunoglobulin M anti-HDV assays are also comparable. However, there are still major differences in sensitivity among some assays for HDAg detection.

Although hepatitis delta virus (HDV) infection can now be detected by a wide range of methods, including immunoblotting (3) and cDNA probes (6, 14), the routine laboratory detection of HDV markers is generally carried out by serological assays (either enzyme immunoassays (EIAs) or radioimmunoassays using in-house reagents [5, 10, 11] or commercial assays, which are mostly EIAs). One or more of three serological markers are used: hepatitis delta virus antigen (HDAg) for acute infection; anti-HDV antibody (anti-HDV) for past infection, epidemiological studies, and chronic infection; and immunoglobulin M (IgM) anti-HDV for the "window" period between the appearance of HDAg and IgG anti-HDV and for indicating chronic HDV infection when present at a high titer. In up to 78% of coinfections, HDAg and IgM anti-HDV can be found concurrently (13).

Previous reports have compared some of the commercial assays for HDV markers by using sera from small numbers of patients (2, 7, 12) and have identified some discrepancies in results obtained with assays for anti-HDV. None have compared the commercial assays for IgM anti-HDV. However, the greatest variation in sensitivity has been reported with assays for HDAg. For example, Dubois and Goudeau, using Organon reagents (Hepanostika anti-delta; Organon Tecnica, Boxtel, Holland) in a study of 22 patients with a positive result for HDAg in the first serum sample, found only 15 of these also positive by the Noctech assay (Deltassay Ag; Noctech, now Cambridge Biotech, Dublin, Ireland); none were reported to be simultaneously positive for both HDAg and anti-HDV (7). Similarly, Bezeaud et al. found that of seven samples positive for HDAg with the Organon reagents, only four were positive by the Pasteur assay

(Deltassay Ag; Diagnostics Pasteur, Paris, France) (2). In contrast, in a study of 135 patients with HDV infection, HDAg was detected by Noctech's Deltassay in sera from 100% of the patients with multiple specimens for a mean duration of 21 days (13); furthermore, the simultaneous presence of IgM anti-HDV and HDAg in 78% of patients with serial specimens indicated the ability of this assay to detect HDAg in immune complexes (13). A later comparison of the Organon and Noctech reagents found that of nine samples positive for HDAg by the Noctech assay, only six were positive with the Organon reagents; the three undetected samples contained IgM anti-HDV, also implying interference by HDAg-IgM complexes (12).

The sensitivity of serological tests for HDV markers, particularly HDAg and IgM anti-HDV, is critical in determining the etiology of severe acute hepatitis, particularly since a significant proportion of patients with acute hepatitis B virus (HBV) surface antigen (HBsAg)-negative and IgM anti-HBV core-positive hepatitis have been shown to have HDV markers (4). We have therefore carried out three separate evaluations on all available commercial EIAs for all three HDV markers with larger panels of stored sera from HDV coinfections and some cases of chronic HDV infection and compared the results with those from in-house assays.

MATERIALS AND METHODS

All serum samples used in this study had been stored at -20°C for various periods (range, 5 months to 9 years), and all were from intravenous drug abusers except for 10 samples from anti-HDV-positive HBsAg carriers of Hungarian origin, 9 samples from HBsAg-positive non-drug abusers, 10 samples with IgM antibody to hepatitis A virus, and 5 samples positive for rheumatoid factor. Apart from the last

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TABLE 1. Comparison of results of HDAg assays with sera from 82 patients

Assay	No. of results				Sensitivity (%)	Specificity (%)	Efficiency (accuracy) (%)
	Positive	Borderline ^a	Negative	False negative			
In-house	42	0	40	0			
Wellcome	42	0	40	0	100	100	100
Pasteur	38 (of 39)	1 ^b	35 (of 35)	0	100	100	100
Noctech	41	1 ^b	40	0	100	100	100
Organon (without Tween 20)	24	1 ^b	57	17	59.5	100	79.3
Organon (with Tween 20)	26	1 ^c	55	15	64.3	100	81.7
Sorin	10	0	72	32	23.8	100	60.9

^a Within 5% below cutoff.^b Same sample was borderline in all three assays.^c Sample different from the one that was borderline in the assays indicated by footnote *b*.

two groups of sera, all samples were HBsAg positive. Serial samples from five patients were arbitrarily included.

The classification of the patients into those with or without HDV infection was originally determined by in-house assays, by examining sera for seroconversion to IgM or total anti-HDV or both and neutralizability with anti-HDV in the case of HDAg positives, by the previous presence of confirmed HDAg or the seroconversion to total anti-HDV in the case of IgM anti-HDV positives, and by the previous detection of confirmed HDAg or IgM anti-HDV or both in the case of total anti-HDV positives.

The kit evaluations were carried out as three separate trials of sensitivity and specificity only by an independent technician with no previous knowledge of the samples. The samples were selected randomly from a wide range of in-house optical densities (OD), and some were arbitrarily included in more than one trial. Different kits in each trial were used simultaneously or within a few days of each other. Assays were carried out and cutoff points were calculated according to the manufacturers' instructions. A result was considered borderline if it repeatedly came within 5% below the cutoff.

Five commercial EIA kits for detecting HDAg were evaluated. A total of 82 HBsAg-positive serum specimens were assayed with each kit, except for the Pasteur kit, for which only 74 of the samples were available because of insufficient amounts of 8 samples. A total of 55 of these samples were previously confirmed to have HDV infection (by in-house assay seroconversions as defined above); 42 of them had HDAg, and the other 13 had either anti-HDV, IgM anti-HDV, or both. Six of the 55 samples were from patients with chronic HDV infection, and 49 were from patients with acute infection (mainly coinfection). Of the 82 HBsAg-positive specimens in the HDAg trial, 27 did not have any HDV markers by in-house assays at any time.

Organon does not supply a separate kit for detecting HDAg. Instead, its instructions state that the anti-HDV kit reagents may be used for detecting HDAg with the addition of a (user-supplied) nonionic detergent. It is also stated that the assay may be carried out without adding any detergent. We therefore carried out the HDAg test as directed with the Organon anti-HDV reagents, using either a final concentration of 3% Tween 20 (as recommended) or no detergent, in parallel assays. Four Organon kits from three different batches were used for HDAg (and anti-HDV) evaluation.

For the evaluation of the six total anti-HDV kits, 81 serum specimens were assayed. Of these, 52 were HBsAg positive, 8 had anti-HBs, and 21 had no HBV markers. Previous in-house tests had confirmed the presence of total anti-HDV

in 36 of the 52 HBsAg positives, and one sample had HDAg alone.

For the IgM anti-HDV evaluation, a panel of 83 serum specimens was tested by three commercial EIAs. Fifty-six of these serum specimens were HBsAg positive, including 11 from carriers of HBV, and 12 had other HBV markers. Fifteen, including five rheumatoid factor-positive serum samples, had no HBV markers. IgM anti-HDV was previously confirmed (by seroconversion as defined above) in 37 of the 56 HBsAg positives by the in-house assay. The IgM anti-HDV assay from Pasteur was not available for this evaluation.

All of the HDAg assays are of the antigen capture sandwich type. The Wellcome (Wellcozyme Anti HD; Wellcome Diagnostics, Dartford, United Kingdom), Pasteur, and Noctech total anti-HDV assays are all sequential sandwich assays. The anti-HDV assay of Organon is a competitive sandwich assay in which the user adds antigen and sample to an antibody-coated well, while that of Abbott (Abbott Anti-delta EIA; Abbott, Chicago, Ill.) is a competitive assay that uses antigen-coated beads. The IgM assays are all of the IgM capture variety. All assays used tetramethylbenzidine chromogen, except those of Pasteur and Abbott, which used *o*-phenylenediamine.

The in-house assays for HDAg, anti-HDV, and IgM anti-HDV and the neutralization assay for confirmation of HDAg, with antiserum kindly supplied by M. Rizzetto, were carried out as previously described (11, 11a, 13).

Sensitivity, specificity, and efficiency were calculated according to the method of Hart (9), and levels of antibody in HDV coinfection and chronic infection were analyzed by Wilcoxon's rank test.

RESULTS

HDAg evaluation. Of the 82 HBsAg-positive serum samples included in the HDAg evaluation, 42 were originally confirmed to be positive for HDAg by in-house assays.

Of the five commercial assays, the Wellcome, Pasteur, and Noctech assays were reactive with all 42 in-house HDAg-positive samples (100% sensitivity). The Organon assay detected a maximum of 26 positives (see below), and the Sorin assay (ETI-DELTAK; Sorin Biomedica, Saluggia, Italy) detected only 10 in-house positives (23.8% sensitivity or 76.2% false negatives). None of the HDAg assays gave positive results with any of the other 40 serum samples in the panel (100% specificity). The efficiency (accuracy) with which the various assays were able to give a true-positive or true-negative result varied from 61 to 100% (Table 1).

TABLE 2. Comparison of results of anti-HDV assays with sera from 81 patients

Assay	No. of results			
	Positive	Borderline ^a	Negative	False positive
In-house	36	0	45	0
Wellcome	35	1 ^b	45	0
Pasteur	36	1 ^c	44	0
Noctech	36	0	45	0
Organon (without Tween 20)	36	0	44	1
Sorin	36	0	45	0
Abbott	36	0	44	1 ^c

^a Within 5% below cutoff.^b Specimen was positive in all other tests.^c Specimen was negative in all other tests but was positive for HDAG.

The Organon assay was carried out with Organon's anti-HDV kit reagents either with or without user-supplied detergent. When these reagents were used for HDAG detection, the number of in-house HDAG-positive samples detected without the addition of detergent was 24 (59.5% sensitivity). When 3% Tween 20 was added to the samples in the test, the total number of positive samples detected increased from 24 to 26 (64.3% sensitivity), with one (different) borderline result by each method. However, not all of these samples were positive by both methods, with only 19 being positive both with and without detergent. This left 14 discrepant results between the two methods. Five of these discrepant serum specimens were positive only when detergent was absent (and one was borderline), and eight serum specimens were positive only when detergent was added. Furthermore, the OD were much lower in all 14 discrepant samples and in most other positives in the Organon assay, with or without detergent, than in the in-house assay and the Wellcome, Pasteur, and Noctech assays (data not shown). Nine other specimens were falsely negative with the Organon reagents, with or without detergent.

Anti-HDV evaluation. Among the 81 serum samples evaluated with the anti-HDV assays, 60 had HBV markers and 37 of these had HDV markers. Of the latter, 36 serum specimens had anti-HDV previously confirmed by in-house assays and 1 had HDAG only. Ten of the anti-HDV-positive serum samples also had concurrent IgM anti-HDV, and eight had concurrent HDAG.

The results with all six commercial anti-HDV assays were similar (Table 2). All of the assays were reactive with all 36 original anti-HDV-positive specimens. Two false-positive results were obtained. One was positive in the Organon assay but negative in all other assays. The other was positive in the Abbott assay and borderline reactive in the Pasteur assay but negative in all other assays. However, this specimen was HDAG positive.

Titration were carried out with each anti-HDV assay on four samples from HBsAg carriers with chronic HDV infection and on three samples from patients with HDV coinfections. All assays showed a similar sensitivity in terms of titers expressed as absolute dilutions, and all showed significantly higher titers ($P < 0.0001$) in chronic carriers, ranging from 1/100 to >1/200,000, than in sera from patients with coinfections, which ranged from neat to 1/100.

IgM anti-HDV assays. Of the 83 specimens evaluated by the IgM assays, 68 were from patients with HBV markers. Of these, 38 were previously determined to be IgM anti-HDV positive by in-house assays.

The Wellcome and Noctech IgM anti-HDV assays showed complete agreement with the in-house assay, with 38 positives, while the Sorin assay showed 36 positives, one borderline positive, and one false negative (that had an OD higher than those of the negatives). None of the assays showed reactivity with the remaining specimens, including 10 serum specimens with IgM anti-hepatitis A virus antibody and 5 specimens positive for rheumatoid factor.

Sera from five patients with chronic HDV infection and five patients with coinfection were titrated. All assays showed a significant difference in IgM anti-HDV titers (expressed as absolute dilutions) between the patients with coinfection and the patients with chronic infection; the titers ranged from 1/100 to 1/5,000 and 1/5,000 to 1/50,000, respectively.

Serial samples. Five patients with a total of 13 serial specimens were randomly included in the evaluations. One sample was anti-HDV positive only, and the remaining 12 samples were from patients who were HDAG positive by the in-house criteria for a period of up to 1 month. All of these samples were similarly positive for HDAG by the Wellcome, Noctech, and Pasteur assays but were negative by the Sorin assay. Assay results varied with the Organon reagents as follows: four samples were reactive for HDAG and four samples were negative with or without detergent; two were reactive only with detergent, and two were reactive only without detergent. There was no correlation between the OD obtained in the in-house assay and the reactivity with the Organon reagents with or without detergent. However, in the only two cases where there were three or more serial specimens, the Wellcome, Noctech, and Pasteur assays detected HDAG in specimens dated 1 and 4 days later than those in which it was detected by the Organon reagents. Three of the 13 serial samples also had anti-HDV by all assays, two simultaneously with HDAG at the late acute stage.

DISCUSSION

In comparing the sensitivity and specificity of new immunoassays, choosing a suitable standard by which to assess such assays is often a problem. There are no panels of standard sera containing HDV markers, and it is therefore necessary to use other criteria. In this study, we used sera for which in-house assays have shown relevant seroconversions or previous appearance of HDAG (as appropriate) in serial specimens, with the presence of such HDAG being confirmed by neutralization assay with an externally tested antiserum.

The greatest differences in the results which we obtained with commercial serological assays for HDV markers were with the HDAG detection kits. These varied from all 42 in-house HDAG positives being reactive in the Wellcome, Pasteur, and Noctech assays (100% sensitivity) to as few as 10 being reactive in the Sorin assay (23.8% sensitivity). The greater sensitivity of the former assays suggested by these data was supported by the finding of HDAG for longer periods in serial samples from two patients in the Wellcome, Pasteur, and Noctech assays but not with the Sorin or Organon reagents. In the case of the Organon test for HDAG, for which the manufacturer suggests the use of its anti-HDV reagents with or without the addition of detergent, it was surprising to find that the total number of HDAG-positive samples detected was hardly changed by the addition of 3% Tween 20. Furthermore, there were 16 discrepant results between the two Organon methods, and the OD were much

lower than those in the in-house, Wellcome, Pasteur, and Noctech assays. Bezeaud et al. (2) have reported that the Organon anti-HDV reagents were more sensitive for the detection of HDAg than the Pasteur HDAg assay, but they used a small number of samples, and Dubois et al. (7) reported that the use of the Organon anti-HDV solid phase wells and conjugate were more sensitive than the Noctech HDAg assay. Our results with the present generation of kits from different manufacturers consistently show that the assays from Wellcome, Pasteur, and Noctech have substantially better sensitivity for detecting HDAg (by almost a factor of two) than the Organon reagents (four kits of three batches each) and four times greater sensitivity than two different batches of Sorin's HDAg assay. The Organon anti-HDV reagents are optimized for anti-HDV detection (as shown by the agreement between the results of the Organon assay and those of all of the other assays in our anti-HDV evaluation); the discrepant results which we obtained with the Organon anti-HDV kit reagents suggest that these reagents are not optimized for HDAg detection. The poor sensitivity of the Organon and Sorin reagents may be due to less stable epitopes being recognized by the reagents and/or nonoptimized extraction conditions.

It is reassuring to note that our results with the anti-HDV evaluation indicate that there are far fewer discrepancies in sensitivity and specificity with the present generation of commercial anti-HDV assays than with those used in studies that were reported in 1988 (7) and in 1989 (2, 12), since all of the commercial assays were reactive with all of the in-house anti-HDV positives. However, there were two false-positive results, one with the Organon assay (for which no reason was apparent) and one with the Abbott assay. The latter was strongly positive in all of the HDAg assays. This phenomenon of false-positive results occurring in some HDAg-containing sera in the Abbott anti-HDV assay was also reported by Dubois and Goudeau (7) and Bezeaud et al. (2). It is almost certainly due to the conjugate becoming saturated with HDAg during the initial concurrent incubation of sample and conjugate on the solid-phase medium and can be overcome by using a sequential incubation (7).

By using in-house assays, it has been previously found in our laboratory and other laboratories that titers of total anti-HDV are higher in cases of chronic HDV infection than after acute infection (1, 13). Our results confirm that high titers are also found with all of the commercial assays tested.

As with the total anti-HDV assays, there was very good agreement between the three commercial IgM anti-HDV assays and the in-house assay and no reactivity with rheumatoid factor. We also noted that titers of IgM anti-HDV were significantly higher in sera from chronically HDV-infected HBsAg carriers than in sera from patients with HDV coinfections, in agreement with our previous findings (13) and with those of Farci et al. (8). This result indicates the usefulness of titrating IgM anti-HDV for detecting chronic HDV infection.

In conclusion, our results indicate that there has been a definite improvement in the sensitivity of commercial assays for the detection of anti-HDV but that sensitivity is still lacking in some assays for HDAg.

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