Evaluation of Assay Methods for Hepatitis-Associated Antigen

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Assay methods for hepatitis-associated antigen (HAA) were evaluated for sensitivity, or reproducibility, or both in a series of three trials in which both research and service-oriented laboratories participated. Agar-gel diffusion (AGD) methods were found to be the least sensitive and reproducible of the commonly employed assay methods. Complement fixation (CF) tests were consistently more sensitive than either AGD or counterelectrophoresis (CEP) methods for detection of HAA. With judicious choice of the antibody reagent, sensitivity of CEP techniques was equivalent to CF methods of HAA detection. None of the three major assay methods (AGD, CEP, or CF) compared in this study were capable of consistently detecting HAA when it was present in relatively low concentrations in human serum.

Australia antigen [hepatitis-associated antigen (HAA)] was discovered by Blumberg by using a modification of the Ouchterlony method of immunodiffusion in agar-gel (2). After this antigen was shown to be associated with serum hepatitis, other methods for detection of the antigen were introduced (4, 8-10, 13-15). The newer assay methods were claimed to afford improved sensitivity, increased rapidity of testing, or greater economy of reagents. Although those workers who introduced new assay methods presented data to document the claimed advantages, no large-scale comparative study of the various methods has yet been reported. Accordingly, at the request of the American Association of Blood Banks, the National Research Council appointed a Committee on Hepatitis-Associated Antigen Tests to evaluate the relative sensitivity of the current assay methods for HAA. An evaluation study consisting of three trials was subsequently set up and was jointly sponsored by the National Research Council, the NIH Division of Biologics Standards, and the Center for Disease Control. This communication reports the results obtained.

MATERIALS AND METHODS

Trial I. Twenty research-oriented laboratories participated in the first and largest trial. The major tests to be compared were double diffusion in agar-gel (AGD), counterelectrophoresis (CEP), and complement fixation (CF). Additional experimental assay methods, including inhibition of passive hemagglutination (PHA) and radioimmunoassay (RIA) were performed by those laboratories currently investigating these particular methods.

Each laboratory was supplied with an identical panel of 120 sera. These sera had been previously assayed for HAA in one or another of the participating laboratories. The sera were submitted to a central point where samples were prepared and code numbers assigned so that the identities of the specimens were not known until after the panel had been tested. Approximately one-third of these sera were negative for HAA. About one-sixth were strongly positive. The remaining half of the panel sera contained low to moderate levels of HAA and were believed to constitute the group of sera which would afford the severest challenge to the various assay methods.

Most of the HAA-positive sera were from apparently normal blood donors. However, a few of these sera were collected from patients with clinically diagnosed serum hepatitis or viral hepatitis type B. The majority of the negative sera were likewise from apparently normal donors, although a few were from patients with infectious hepatitis or hepatitis due to nonviral causes.

Each laboratory was supplied with an antiserum to HAA produced in guinea pigs (contributed by R. H. Byrne, National Institute of Allergy and Infectious Diseases) and one of two HAA antisera of human origin (contributed by D. J. Gocke, College of Physicians and Surgeons of Columbia University and S. L. Rivers, The American National Red Cross, Atlanta Chapter). Each laboratory also received an
HAA-positive reference serum. The plan called for the panel of sera to be tested with each of the two supplied antisera and with at least one additional antiserum routinely used in each laboratory. These additional antisera were of both human and non-human origin.

Each of the laboratories was asked to perform AGD, CEP, and CF tests on the panel of sera, but it was not always possible or appropriate for them to do so. Not all laboratories felt competent in each method and, furthermore, one of the supplied antisera of human origin was not reactive in the CF test. As a result, an unequal number of tests was performed by each method. The mean number of tests per serum was 76 for AGD, 67 for CEP, and 33 for CF. Two laboratories used PHA and two used RIA methods to detect HAA in the panel of sera.

**Immunodiffusion methods.** The AGD tests were used by 19 of the 20 laboratories and were performed basically according to the method of Blumberg and Riddell (2). However, no two laboratories used exactly identical procedures, and one laboratory used three different AGD techniques. Agarose was used as a substrate in 15 of the 21 variations of the AGD method, with concentrations varying between 0.6 and 1.1% Ionagar no. 2 (0.7, 0.9, and 1.0%) used in three. One laboratory each used the following: 1.0% special agar (Noble), 1.0% purified agar, and a commercially prepared plate of unknown composition. Twelve of the laboratories added NaCl to the gel substrate at concentrations of 0.077 to 0.15 M, five added NaCl, and one of the supplied antisera. No buffering preparations of unknown salt content. Six laboratories added no buffering compounds to the gel substrate. Eight laboratories buffered the substrate with 0.01 to 0.001 M tris(hydroxymethyl)aminomethane (Tris) to which was added 0.001 M ethylenediaminetetraacetate (EDTA), two used phosphate buffers (0.1 M), and two used barbital buffers (0.0375 and 0.05 M). The buffering systems of three commercial preparations were not known. The pH values of the prepared substrates ranged from 6.2, when no buffer was used, to 8.6 with a barbital buffer. The Tris-buffered substrates ranged from pH 7.0 to 8.0 (usually 7.6). Three laboratories added protamine sulfate (0.1 mg/ml) to the substrate, and two added sodium azide.

All laboratories used a well pattern consisting of a central well surrounded by six equally spaced wells. The distance (center to center) from the center well to each peripheral well varied from 3 to 10 mm, the most common distances being 5 to 7 mm. Except for a few instances, the well diameters were 2.0 to 3.0 mm.

One laboratory used a template with wells of 25-µl capacity on the surface of the gel rather than cutting wells into the substrate. The volume of serum or reagent added to each well varied from 4 to 100 µlitters. In one laboratory, the test specimen was concentrated approximately tenfold with polycrylamide gel before it was added to the well. Eighteen of the laboratories filled the well once only. The remaining two laboratories refilled the well containing the test serum. Two laboratories incubated the test at 37 C; 18 methods called for incubation at room temperature (22 to 25 C). The tests were read at various intervals beginning at 2 hr and ending at day 7, usually without staining. Two laboratories made an additional reading after staining, one with azo-carmine, the other with ponceau.

**Counterelectrophoresis methods.** Sixteen of the 20 laboratories performed CEP tests. Two laboratories used more than one CEP technique. The procedures were based on the techniques described by Gocke and Howe (4). Two laboratories in each laboratory had introduced variations so that no two laboratories performed the test in an identical manner. All used agarose (0.85 to 1.0%) as a substrate. In 11 of the 20 variations, the substrate was buffered with barbital (0.037 to 0.05 M, pH 8.2 to 8.8) and the same buffer at the same or slightly reduced concentrations was used in the reservoirs. An acetate-sodium barbital buffer (0.04 to 0.1 M, pH 8.2 to 8.6) in both substrate and reservoir was used in six techniques. One laboratory used a 0.05 M Tris-barbital-sodium-barbital buffer, pH 8.8. Another used a 0.01 M Tris buffer, pH 7.6, in the gel and a 0.05 M barbital buffer in the reservoir.

The well patterns consisted of two to four parallel double rows of wells. Each pair of wells was spaced 5 to 11 mm apart (center to center); most techniques utilized a 10-mm spacing. The well diameters varied from 2.0 to 5.0 mm, with 3 mm being the most common size. In six instances the well which received the antiserum was 0.5 to 1.5 mm smaller in diameter than the test serum well. The volume of test serum used varied from 5 to 40 µlitters, with 20 µlitters being most common.

The bridge distance (wrick to wick) varied from 3.0 to 8.0 cm. Wicks used were of chromatographic paper, terry cloth, or dacron felt.

The current applied across the bridge varied from 3.3 to 145 ma. Ten of the procedures maintained a constant amperage. The voltage used varied from 50 to 900 v. Eleven procedures maintained a constant voltage. The current was applied for 1 to 2.5 hr, the majority of the techniques utilizing a 1-hr period.

Three laboratories used circulating water to maintain the temperature of the reaction at 19 to 23 C. One laboratory used a thermoelectric plate to maintain a temperature of 21 C; the remainder of the laboratories conducted the run at room temperature (21 to 27 C). All but one laboratory examined the plates for precipitin lines immediately after the electric current was stopped. All but two laboratories made an additional reading at 24 hr after the run and occasionally at 48 hr also. All laboratories read the unstained reaction. Three laboratories made an additional reading after staining the plates.

**CF methods.** Twelve laboratories performed CF tests, all with micro methods (3, 6, 7, 10, 12). The major variations involved complement dosage and incubation periods. The dose of complement varied from 1.7 100-% units to five 50-% units. Ten of the laboratories incubated the test overnight at 4 C before adding sensitized cells. One laboratory incubated the test at room temperature for 2 hr and another laboratory incubated for 1 hr at 37 C before
addition of the hemolytic system.

Other methods. The methods used for RIA and PHA have been described (14, 15).

Laboratories were asked to report results of AGD and CEP tests as either positive or negative. Because of the nature of the CF test, results were reported as titers of antigen. To permit a limited comparison of antigen titers by the various assay methods, each laboratory was asked to titrate, by each method, the first 10 specimens found to be positive.

Participating laboratories. The following participated in Trial I: Harvey J. Alter, Paul V. Holland, and Robert H. Purcell, Clinical Center Blood Bank and Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Md. 20014; William H. Bancroft, Walter Reed Army Institute of Research, Washington, D.C. 20012; Lewellys F. Barker, Laboratory of Viral Immunology Division of Biologics Standards, NIH, Bethesda, Md. 20014; David J. Gocke, Columbia University College of Physicians and Surgeons, New York, N.Y. 10032; Martin Goldfield and Henry C. Black, New Jersey State Health Department, Trenton, N.J. 08625; George F. Grady, Massachusetts State Health Department, Boston, Mass. 02130; Tibor J. Greenwalt and Jack J. Levin, The American National Red Cross, National Headquarters, Washington, D.C. 20006; Milford H. Hatch, Enteric Virology Unit, Center for Disease Control, Atlanta, Ga. 30333; Saul Krugman and Joan Giles, New York University School of Medicine, Department of Pediatrics, New York, N.Y. 10016; Edwin H. Lennette and Nathalie J. Schmids, California State Health Department, Berkeley, Calif. 94704; W. Thomas London and Baruch S. Blumberg, The Institute for Cancer Research, Philadelphia, Pa. 19111; James E. Maynard, Center for Disease Control, Ecological Investigations Program, Phoenix, Ariz. 85014; Robert B. Pennell, Blood Research Institute, Boston, Mass. 02115; Robert L. Peters, University of Southern California School of Medicine, Department of Pathology, Los Angeles, Calif. 90007; Alfred M. Prince, The New York Blood Center, New York, N.Y. 10021; Shirley L. Rivers, Atlanta Regional Red Cross Blood Center, Atlanta, Ga. 30308; Richard E. Rosenfield, The Mount Sinai Hospital Blood Bank, New York, N.Y. 10029; J. F. Sgouris, Michigan State Health Department, Lansing, Mich. 48914; Girish N. Vyas, University of California, San Francisco Medical Center, Department of Clinical Pathology and Laboratory Medicine, San Francisco, Calif. 94122; and John H. Walsh, Veterans Administration Center, Los Angeles, Calif. 90073.

Trial II. The second trial was designed to evaluate the sensitivity and reproducibility of the CEP test in laboratories performing the test as a routine service. Five laboratories participated. In addition, one laboratory which participated in Trial I was asked to serve as a referee laboratory.

Each laboratory was given a panel of 100 coded serum samples which consisted of duplicates of 50 sera. Thirty-six of the sera had also been part of the panel used in Trial I. On the basis of the original trial, 26 of these 36 sera were considered to be HAA positive and 10 HAA negative. Fourteen additional sera not previously used in the first trial were added to this panel; 10 were considered positive on the basis of previous CF or CEP tests. Thus, the panel consisted of duplicates of 36 HAA-positive and 14 HAA-negative sera.

Each laboratory was asked to use an antiserum which it routinely employed for CEP tests and to perform the tests within the following specifications. (i) A power source should be used which supplies a constant current of 5 to 6 ma per lateral inch. (ii) Buffer in the reservoirs should be 0.05 M barbital buffer, pH 8.2 to 8.8, and the electrodes should run the entire length of the reservoir. (iii) A substrate of 0.85 to 1.0% agarose buffered to pH 8.2 to 8.8 with 0.05 M barbital buffer should be used. (iv) Wells should be distributed along the length of the slide in three columns of 10 paired wells with an edge-to-edge distance of 3 to 4 mm. Ten mm should be allowed between the paired columns and wells should be 3 to 5 mm in diameter. (v) Wells should be filled with 10 to 25 μlitters of antiserum or test serum, and the surface of the reagent should be level with the agar surface. Wells should not be refilled. (vi) Current should be applied for 1 to 1.5 hr. (vii) Plates should be read immediately after termination of the current flow. Readings should be made with oblique light against a black background and should be made by two independent observers. (viii) Control positive and negative sera should be included on each plate.

Participating laboratories. The following participated in Trial II: William L. Bayer, Community Blood Bank of the Greater Kansas City Area, Inc., Kansas City, Mo. 64111; Milford H. Hatch, Enteric Virology Unit, Center for Disease Control, Atlanta, Ga. 30333; F. Blaine Hollinger, Department of Virology and Epidemiology, Baylor College of Medicine, Houston, Tex. 77025; Jessica Lewis, Central Blood Bank of Pittsburgh, Pittsburgh, Pa. 15219; Jack Lubin, Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Miami Beach, Fla. 33140; and Morris Schaeffer, Bureau of Laboratories, New York City Department of Health, New York, N.Y. 10016. (Note: Names are arranged alphabetically, and the order here does not correspond to laboratory numbers in Table 5.)

Trial III. The third trial was designed to compare the relative sensitivity and reproducibility of the AGD and CEP methods for detection of HAA. Each laboratory was supplied with two differently coded panels of duplicates of the same 50 sera. This was to avoid a bias possibly present in Trial I where each method was performed on the same sample. All HAA-positive sera used in Trial III were among those that had been included in the panel for Trial I.

Ten of the 50 sera were considered negative for HAA. The remaining 40 sera were HAA positive and included some with minimal amounts of antigen and some in which antigen was more easily detected in Trial I. One panel was to be tested only by AGD and the other panel only by CEP. The CEP tests were to be performed either within the specifications set for
the CEP tests in Trial II or with commercially available kits. No specifications were set for the AGD tests. However, the techniques used fell within the parameters of the AGD tests as performed in Trial I or utilized commercially available kits or materials.

The antisera used in the trial were those routinely employed by the participating laboratories for the detection of HAA.

Nine laboratories offering HAA assay as a routine diagnostic service participated in Trial III. In addition, one laboratory which had participated in Trial I and which was engaged in both research and routine diagnostic service acted as a referee laboratory.

**Designation of positive and negative samples.** A sample was defined as HAA positive or negative on an arbitrary basis because there is no test generally accepted as having absolute sensitivity for detecting HAA. The classification of a specimen as HAA positive or negative was determined by the consensus of the 177 to 187 separate tests performed in these trials on each serum. If only 5% or less of all tests were reported as positive, the serum was considered to be HAA negative. Assuming that no real differences existed in the sensitivity of AGD, CEP, and CF tests, the distribution of all positive results should be random among the three tests. Two-by-three contingency tables were constructed for results of AGD, CEP, and CF tests in Trial I on individual sera. In those instances where the total number of positive results fell below 5% of the tests applied to the specimen, the false positive rates (based on the consensus defined above) of the three tests appeared to be equivalent, and the positive results were generally randomly distributed among the AGD, CEP, and CF tests. Two of the 42 sera constituting this group provided exceptions. One of these two sera was from a volunteer who had previously received the MS2 hepatitis virus, type B. The other was the serum of a jaundiced patient diagnosed as suffering from halothane-induced hepatitis. However, these sera were arbitrarily classed among the HAA-negative group because the per cent of positive tests was still lower for these two than for others in the group which showed a random distribution of positive results. In addition, the CF titers observed with these two specimens varied widely from 1:4 to 1:1024.

The CF titers on the positive specimen considered to have the least amount of HAA were uniformly low (1:2 to 1:16). In general, the geometric-mean CF titer of a specimen increased as the percentage of positive test results increased. Of the 78 sera constituting the HAA-positive specimens of the Trial I panel, 19 were apparently so strongly positive that few negative results were observed by any test method. The negative results on these 19 sera were randomly distributed among the AGD, CEP, and CF tests.

The remaining 59 HAA-positive sera were the more "difficult" sera to identify; they gave positive results in 6.6 to 91.8% of all tests applied to these specimens in Trial I. Positive results for these 59 sera, except in a few cases, were nonrandomly distributed between the AGD, CEP, and CF tests.

**Participating laboratories.** The following participated in Trial III: C. Barrie Cook, Fairfax Hospital Laboratory, Falls Church, Va. 22046; Frank Ellis, Southeastern Michigan Red Cross Center, Detroit, Mich. 48232; Inez Elrod, Piedmont Carolina Blood Center, Charlotte, N.C. 28203; Eloise R. Giblett, King County Central Blood Bank, Inc., Seattle, Wash. 98104; Kathleen Heidelberger, Department of Pathology, University of Michigan, Ann Arbor, Mich. 48104; Fred Holtz, St. Joseph Mercy Hospital, Ann Arbor, Mich. 48111; Charles Huggins, Blood Bank and Transfusion Service, Massachusetts General Hospital, Boston, Mass. 02114; John W. Palmer, Hyland Division, Travelon Laboratories, Inc., Costa Mesa, Calif. 92626; James D. Perry, Community Blood and Plasma Service, Inc., Los Angeles, Calif. 90057; and Shirley L. Rivers, Metropolitan Atlanta Chapter, American National Red Cross, Atlanta, Ga. 30308. (Note: Names are arranged alphabetically and the order here does not correspond to laboratory numbers in Table 6.)

**RESULTS**

**Trial I: agar-gel diffusion tests.** The panel of sera was subjected to 74 separate AGD tests. In the few instances where the results were read without staining and reread after staining, the panel was considered to have been tested twice. An occasional serum was retested after additional dilution. The 74 AGD tests identified from 0 to 96.2% of the 78 HAA-positive sera with a median of 65.4% (Table 1). The test which identified no positive specimens used an antiserum which had become bacterially contaminated. The laboratory identifying 96.2% of the positive specimens concentrated the specimens tenfold with lyphogel. When these two "outliers" are not considered, the AGD tests were found to have identified between 20.5 and 87.2% of the positive speci-

| Table 1. Comparative sensitivity of agar-gel diffusion, counter electrophoresis, and complement fixation tests for detection of hepatitis-associated antigen a |
|---------------------------------------------------------------|
| **Test** | No. tests per sample | Range of per cent correctly identified | Median | Range of per cent correctly identified | Median |
| AGD | 74 | 0 to 96.2 | 65.4 | 0 to 89.8 | 53.3 |
| CEP | 66 | 20.5 to 97.2 | 82.7 | 3.4 to 96.6 | 78.0 |
| CF | 34 | 42.3 to 98.7 | 91.0 | 32.0 to 98.3 | 86.4 |

* Abbreviations: HAA = hepatitis-associated antigen; AGD = agar-gel diffusion; CEP = counter electrophoresis; CF = complement fixation.
mens.

The AGD tests identified from 0 to 89.8% of the 59 more difficult positive sera, with a median of 53.3% (Table 1). A comparison of the AGD methods used on these 59 sera furnishes little information regarding the variabilities of the AGD test. The laboratory which concentrated the serum tenfold identified 87.6% of the 59 more difficult sera and was the only laboratory using purified agar as a substrate. When unconcentrated serum was used, a mean of 49.5 of the 59 difficult sera were identified as HAA positive by those laboratories using agarose, 47.5% by the laboratory using Noble agar, and 39.7% by those using Ionagar.

Laboratories using either no buffer or phosphate buffer in the substrate identified fewer of the more difficult sera than those using Tris or Veronal buffers. Tests using a pH between 7.0 and 8.2 identified more positive specimens than tests performed at lower or higher pH values.

The number of positive specimens identified was not apparently influenced by the distance between wells containing the antiserum and test sample, within the range of 3 to 6 mm. Tests using a well separation greater than this identified fewer of the difficult sera.

The volume of test sample used, except when concentrated serum was employed, did not influence the results. No apparent relationship was observable between the results obtained and the combination of volume of test serum used and well distances. Staining the reaction did not increase the efficiency of identifying positive reactions.

The AGD tests which used the two human source antisera supplied with the test panel identified a mean of 59.3% (range = 37.3 to 81.4%) and 53.4% (range = 27.1 to 86.4%) of the 59 more difficult HAA-positive specimens. A mean of 58.4% (range = 28.8 to 81.4%) of these specimens were correctly identified as HAA positive in AGD tests using various other human source antisera. Tests employing the guinea pig antiserum supplied with the panel identified a mean of 35.3% (range = 0 to 76.3%) of these positive sera. Additional tests with other nonhuman source antibody identified a mean of 42.1% (range = 5.1 to 64.4%) of this portion of the panel. Antibody prepared in guinea pigs was generally less efficient in the AGD test than antibody prepared in rabbits. However, with one guinea pig reagent, 64.4% of the specimens in the more difficult group were properly identified.

Experience does not readily lend itself to measurement. However, those laboratories which had accrued extensive experience working with the AGD test for HAA performed better than those laboratories becoming involved more recently in HAA assay.

The laboratory obtaining the best AGD test results used 1% purified agar containing 0.1 M NaCl buffered to pH 7.6 with Tris buffer. Wells were spaced 4 mm apart and held 30 to 40 lites of concentrated serum. The serum was concentrated about tenfold with polyacrylamide gel. The tests were kept at room temperature and were read first after an incubation period of 18 hr and again at 7 days.

Among those laboratories not utilizing concentrated serum, the best results were obtained by a laboratory using 1.1% agarose without NaCl buffered at pH 8.2 with 0.0375 M barbital. The wells were spaced 6 mm apart and held 7 lites of serum. The tests were incubated at 25 C and read at 24 and 48 hr.

Counterelectrophoresis tests. The panel of sera was tested in 66 separate CEP tests. Tests on an occasional specimen were repeated. From 20.5 to 97.2% of the 78 HAA-positive sera were correctly identified in the various CEP tests (median = 82.7%).

The CEP tests correctly identified from 3.4 to 96.6% (median = 78.0%) of the 59 HAA-positive sera constituting the more difficult group (Table 1).

The parameters of the CEP tests did not vary as widely between laboratories as did those of the AGD tests.

The various buffers and pH ranges used in the various tests did not appear to influence the results observed between the various laboratories. Similarly, results did not appear to vary according to the volume of test serum used, the amperage applied, or the distance between wells. The two laboratories which read results both before and after staining identified more positive HAA specimens after staining. However, each of these laboratories still identified fewer of the positive specimens than the mean of all laboratories performing the CEP tests.

The laboratory identifying the highest number of HAA-positive specimens used a method employing 0.01 M Tris buffer, pH 7.6, in the substrate and barbital buffer in the reservoirs (1). The wells were spaced 8 mm apart and held 25 lites of test serum or antiserum. A current of 30 ma was applied for 1.5 hr. Results were read immediately after termination of the current flow and again after overnight incubation at room temperature.
CF tests. Thirty-four separate CF tests were applied to the panel of sera and identified from 42.3 to 98.7% of the 78 HAA-positive sera (median = 91.0%). The CF tests correctly identified from 32.0 to 98.3% of the 59 more difficult HAA-positive sera (median = 86.4%) (Table 1).

No notable differences were observed in the results obtained with the various CF techniques. Three laboratories encountered more than one anticomplementary serum in the panel, but these results did not appear to be related to any particular factor of the test such as complement dosage or primary incubation period. No particular sera could be identified as being anticomplementary in the majority of the CF tests.

Fourteen sera were titrated for their HAA content by AGD, CEP, and CF methods. Geometric-mean titers are shown in Table 2. The CF titers were the highest with a mean range of five to eight times the titers observed in the CEP tests. The CEP test titers in turn were approximately five to six times those obtained in the AGD tests.

The results obtained on the 59 HAA-positive sera which presented the more difficult problem in proper identification were examined in greater detail (Table 3). A significantly greater proportion of these sera were correctly identified by CEP and CF tests than by AGD tests, regardless of the antiserum used (Table 3). A significantly greater proportion of these sera were diagnosed as HAA positive by CF tests than by CEP tests using the guinea pig antiserum supplied to all participating laboratories (Table 3). However, the differences between CEP and CF tests using one human source antibody and the collective additional antisera were not significant at the 5% significance level and, hence, appear to be equivalent (Table 3).

The antiserum used in CF tests did not significantly influence the proportion of HAA-positive sera correctly identified. The guinea pig antiserum identified a significantly lower proportion of the positive sera when used in AGD tests than did the two human antisera or the group of additional antisera (Table 3). No significant differences were observed in the proportion of the 59 sera correctly identified as HAA positive in AGD tests using the two human or the additional antisera (Table 3). CEP tests using human antiserum no. 1 correctly identified a significantly greater proportion of the difficult sera than those CEP tests using any of the other antibody sources.

Comparison of antisera. To obtain some additional insight into the effect that the choice of antiserum had upon the results obtained from laboratory to laboratory, the results on the entire serum panel from every laboratory using a specific method-antiserum combination were compared with results from every other laboratory using the same method-antiserum combination. Table 4 shows that human serum no. 1 afforded the most consistent results from laboratory to laboratory, regardless of the assay method in which it was used. The results obtained with guinea pig antiserum compared favorably in the different laboratories when used in CF tests, but agreement between laboratories was poorer when this antiserum was used in AGD tests. Human serum no. 2 produced more consistent results in CEP tests than in AGD tests.

False positive reactions were observed in 100 of the 7,465 tests performed on the 42 HAA-negative sera. Usually only one or two false positive results were recorded by any laboratory. However, a significantly higher number of false positives (10 to 22 per laboratory) was observed by each of three laboratories with either AGD, CEP, or CF methods. If the results of these "outliers" are not considered, the false positive rate approximates 0.6% by each of the three methods.

False negative rates were calculated only on the 19 strongly positive specimens. When "outliers" are eliminated, the false negative rate for AGD was 3.47% and for CEP 2.52%; with the CF method, no false negative results were recorded.

### Table 2. Geometric-mean titers of selected HAA-positive sera as measured by various assay methods

| Specimens | AGD | CEP | CF |
|-----------|-----|-----|----|
| 2         | 9.57| 59.71| 441.99|
| 3         | 10.08| 46.70| 443.91|
| 5         | 1.14| 5.28| 34.80|
| 6         | 0.55| 1.47| 5.72|
| 7         | 6.26| 40.32| 365.86|
| 8         | 0   | 1.05| 4.61|
| 9         | 1.88| 14.93| 50.11|
| 10        | 4.46| 29.08| 177.14|
| 11        | 0.51| 1.49| 4.34|
| 12        | 10.94| 48.50| 501.36|
| 13        | 0.51| 0.57| 2.98|
| 14        | 1.41| 7.55| 41.71|
| 15        | 1.90| 15.43| 108.74|
| 20        | 3.89| 24.05| 162.34|

*Abbreviations: HAA = hepatitis-associated antigen; AGD = agar-gel diffusion; CEP = counter electrophoresis; CF = complemen t fixation.
**Table 3. Results of hepatitis-associated antigen (HAA) assay on 59 HAA-positive sera of high to moderate diagnostic difficulty**

| Antiserum used              | Test method             |
|-----------------------------|-------------------------|
|                             | Agar-gel diffusion (AGD) | Counterelectrophoresis (CEP) | Complement fixation (CF) | AGD vs. CEP | AGD vs. CF | CEP vs. CF |
| Guinea pig                  | 437/1235*               | .3530*                        | 605/1002*               | .6038*       | *          | *          |
| Human no. 1                 | 455/787                | .5537*                        | 675/826                | .8172        | *          | NS         |
| Human no. 2                 | 220/413                | .5337*                        | 258/354                | .7288        | Not done   | *          |
| All additional antisera     | 1051/1938              | .5423                         | 1284/1688              | .7807        | *          | NS         |
| Guinea pig vs. human no. 1  | *                     | *                             | NS                     | *            | NS         |
| Guinea pig vs. human no. 2  | *                     | *                             | NS                     | *            | NS         |
| Human no. 1 vs. human no. 2 | NS                    | *                             | NS                     | *            | NS         |
| Human no. 1 vs. additional human no. 2 | NS | *                             | NS                     | *            | NS         |
| Human no. 2 vs. additional human no. 2 | NS | NS                             | NS                     | *            | NS         |

* Number of positive results per total number of tests applied.
* Proportion of positive results to total tests.
* Significant difference between the two proportions at the 5% joint significance level.
* NS = Nonsignificant difference between the two proportions at the 5% joint significance level.

**Table 4. Interlaboratory agreement for selected test method-antiserum combinations**

| Antiserum | AGD | CEP | CF |
|-----------|-----|-----|----|
| Hu 1      | 91  | 88.5| 76.3|
| Hu 2      | 15  | 77.5| 86.5|
| GP        | 210 | 91.2| 83.2|
| Hu 1      | 92  | 86.5| 87.5|
| Hu 2      | 15  | 83.2| 87.7|
| GP        | 136 | 87.7| 9.3 |

* Abbreviations: Hu 1, human serum no. 1; Hu 2, human serum no. 2; GP, guinea pig serum; AGD, agar-gel diffusion; CEP, counterelectrophoresis; CF, complement fixation.

**Hemagglutination-inhibition method.** The panel of sera was tested a total of five times in two laboratories by the passive hemagglutination-inhibition method. Three hundred and seventy-nine positive reactions were recorded among the 390 tests performed on the 78 HAA-positive sera in the panel, a positive reaction rate of 97.2%. Two hundred and eighty-four of the 295 tests (96.3%) performed on the 59 difficult sera were recorded as HAA positive. No false positive reactions were observed. No negative reactions were seen among the tests applied to the 19 strongly HAA-positive sera in the panel.

**Radioimmunoassay method.** The panel of sera was tested once in each of two laboratories by the RIA method. One hundred and forty-eight (96.1%) positive reactions were observed among the 154 tests applied to the 78 HAA-positive sera. One hundred and eighteen RIA tests were applied to the 59 difficult sera, and 112 (94.9%) of these were recorded as HAA positive. Eight positive reactions were observed among the 82 tests applied to the HAA-negative sera in the panel, a false positive reaction rate of 9.8%. Both of the RIA tests on two of the specimens gave positive results. Further testing has indicated that these two specimens, both from the same individual, are definitely HAA positive, as is a third serum, also from the same individual, which was scored negative by both RIA tests. The antigen content of these sera is clearly below the threshold for detection by AGD, CEP, and CF. Otherwise, positive reactions among the HAA-negative sera were observed in only one or the other RIA test. No negative reactions were observed among the 19 strongly HAA-positive
sera in the panel.

**Trial II.** Eight hundred and sixty-four CEP tests were performed on the 36 HAA-positive sera included in the Trial II panel. Each of these sera was correctly identified in at least two of the five participating laboratories. Overall, 660 (76%) of the tests on these 36 sera were interpreted as being HAA positive. The individual tests identified from 19.4 to 90.3% of the positive samples (Table 5).

There did not appear to be a difference in sensitivity which could be attributed to the antiserum used. The National Heart and Lung Institute interim reagent was used in four laboratories, with sensitivities of 19.4, 61.1, 83.3, and 90.3%. The other antiserum used correctly identified between 75 and 90.3% of the positive specimens. Reproducibility, or the ability of a laboratory to obtain the same result on both samples of the same serum, ranged from 82 to 100% of the 50 different sera. The mean reproducibility of the laboratories' results was 93.5%.

Four of the 336 tests on the 14 HAA-negative sera in this panel were interpreted as positive for an overall 1.2% false positive rate. The four false positive tests were observed by two laboratories.

**Trial III.** Each of the 50 sera in the Trial III panel was tested for HAA by the nine laboratories a total of from 19 to 20 times by AGD and 23 to 24 times by CEP.

Four hundred and ninety-one of the 796 AGD tests (61.6%) performed on the 40 HAA-positive sera were interpreted as positive. The various AGD tests correctly identified from 42.5 to 85.0% of the HAA-positive specimens in the panel (Table 6).

The antisera used in the AGD tests, whether from commercial or noncommercial sources, did not influence the results to any noticeable extent. Two laboratories happened to use the same lot of one commercially available antiserum. One laboratory identified 85.0% of the HAA-positive specimens, whereas the other laboratory identified 42.5%.

Four of the 200 AGD tests performed on the 10 HAA-negative sera in this panel were interpreted as positive, giving a false positive rate of 2%. Two of the nine laboratories performing AGD tests accounted for the false positive results. Both used antiserum from a commercial source.

Of the 956 CEP tests performed on the 40 HAA-positive sera, 691 (72.3%) were interpreted as positive. The tests, as performed by the individual laboratories, correctly identified

| Table 6. Sensitivity and reproducibility of agar-gel diffusion and counterelectrophoresis methods for detection of hepatitis-associated antigen (Trial III) |
|---------------------------------------------------------------|
| Laboratory | Agar-gel diffusion | Counterelectrophoresis |
|------------|-------------------|------------------------|
|            | Sensitivity (%)   | Reproducibility (%)    | No. of false positive results (%) |
|            |                   |                        |                                    |
| 1          | 85.0              | 100                    | 0                                   |
| 2          | 52.5              | 80                     | 0                                   |
| 3a         | 55.0              | 78                     | 1                                   |
| 3b         | 62.5              | 84                     | 0                                   |
| 4          | 73.8              | 98                     | 0                                   |
| 5          | 42.5              | 84                     | 0                                   |
| 6          | 53.9              | 80                     | 3                                   |
| 7          | 82.5              | 100                    | 0                                   |
| 9c         | 70.0              | 84                     | 0                                   |
| 9d         | 67.5              | 94                     | 0                                   |
| 10         | 53.8              | 90                     | 0                                   |

* Per cent of 77 to 80 hepatitis-associated antigen positive samples correctly identified.
* Per cent of agreement of results on 47 to 50 identical sample pairs.
* Twenty negative samples were tested.
* Repeat tests within a laboratory using different antibody reagents are indicated by the letters a and b.
* Two different laboratories within same institution are indicated by the letters c and d.
from 51.3 to 85.0% of the HAA-positive specimens in the panel.

The differences observed could not be attributed to the choice of commercial or noncommercial antisera or agarose plates. However, two laboratories using the same lot of one commercially available antisera correctly identified the lowest and next lowest proportions of the HAA-positive specimens.

Twenty-six of the 240 CEP tests performed on the 10 HAA-negative sera were interpreted as positive, giving a 10.8% false positive rate. Four of the ten laboratories performing CEP tests accounted for these false positive results. False positive results were observed with both commercial and noncommercial reagents.

Reproducibility ranged from 78 to 100% (mean = 87.2%) in the AGD tests. In the CEP tests, reproducibility averaged 92.8%, with a range of 82 to 100%.

The reference laboratory performed RIA and coated-latex particle agglutination tests, as well as AGD and CEP, on this panel of sera. Five of 39 RIA tests applied to the HAA-negative specimens were reported as positive. Only one of 150 RIA tests on the HAA-positive specimens was reported as negative. No false positive results were obtained in the latex particle agglutination tests. This test correctly identified 87.5% of the HAA-positive specimens.

**Laboratory comparisons.** Forty-seven of the HAA-positive sera included in the test panels of Trial I were also used in Trial II or Trial III, or both. Nineteen of these were included in all three trials. The primarily research-oriented laboratories participating in Trial I performed 3,236 CEP tests on these sera, reporting 2,455 (75.9%) of these as HAA positive. The blood bank and service-oriented laboratories which participated in Trials II and III performed a total of 1,580 CEP tests on these same sera, obtaining 1,156 (73.2%) positive results.

**DISCUSSION**

The results of these studies are in general agreement with comparative sensitivity studies reported by others (1, 5, 11).

The panels of HAA-positive sera were selected to include a relatively high proportion of those sera which could present diagnostic difficulties. The panels were therefore not necessarily representative of "easy" and "difficult" sera normally encountered in routine blood banking operations. At the same time, the selection of positive sera was biased toward those that could be detected by at least one of the three major methods compared in these trials. However, none of the sera included in the panels as HAA negative on the basis of preliminary AGD, CEP, and CF tests was subsequently judged to be HAA positive by these methods after receiving the extensive testing which these trials afforded.

It is clear that the AGD method as commonly performed is the least sensitive of the three major methods compared. The CF test was consistently the most sensitive of the three methods. However, with proper selection of the antibody to be used, the CEP test can be as sensitive as the CF test. With the reagents used in Trial I, the CEP test appeared to be least influenced by the choice of antibody. The CEP test is most dependent on the choice of antibody to obtain maximum sensitivity.

It was not within the scope of this study to make systematic comparisons of the variations used within each method. In fact, the procedures used were those having been found by the participating laboratories to give supposedly acceptable results. As such, the variations used encompassed a rather narrow range of possible variations. Aside from the antibody used, the results obtained in the different laboratories by any one method usually could not be directly attributed to any single variable within the method. For example, concentration of the serum to be assayed for HAA apparently increases the sensitivity of the AGD method. However, one laboratory was capable of obtaining almost equivalent sensitivity in the AGD test without concentration of the test samples. The effect of minor differences in methods on CEP test results when the same antisera is used was quite striking in Trial II, as shown by the results with the National Heart and Lung Institute reagent.

Agreement between laboratories was closer with the CF test than with CEP or AGD tests. This may reflect the greater ease with which a positive or negative result is detected by CF than by precipitin techniques. The precipitin tests are technically simpler to perform, but they require careful scrutiny to detect weak or faint reactions. Thus, the lesser interlaboratory agreements observed in the precipitin-type tests may represent "human error" rather than a fundamental characteristic of the techniques per se. This is especially noted in Trial III, where the highest and lowest sensitivities in the CEP test were obtained by two laboratories using the same antibody preparation.

The CEP test appears to also be superior to the AGD test in respect to reproducibility.
Again, this could be attributed to the fact that precipitin lines obtained in CEP tests are generally heavier than those observed in AGD tests and, therefore, fewer reactions will escape the scrutiny of the observer reading the test. Insufficient assays performed by radioimmunoassay, passive hemagglutination-inhibition, and latex particle agglutination were included in this study to make valid comparisons with AGD, CEP, and CF methods for assay of HAA. However, the tests that were performed certainly suggest that AGD, CEP, or CF do not offer the ultimate in sensitivity for detection of HAA.

The comparison of the CEP results in Trial I with Trials II and III indicate that service-oriented laboratories can be expected to obtain levels of sensitivity equivalent to those obtained by research laboratories.

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