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Evaluation of enzyme immunoassay (EIA) as a screening method for hepatitis B markers in an open population

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SUMMARY

Commercially available kits for detection of hepatitis B surface antigen (HBsAg) and hepatitis B surface antibody (anti-HBs) by enzyme immunoassay (EIA) were evaluated in American Samoa during a public health programme to eliminate the transmission of hepatitis B. The first 19184 serum specimens obtained, representing 68% of the total cooperating population, were initially tested for anti-HBs, and those without detectable antibody were tested for HBsAg. All the antigen-positive serum samples, and a selection of the antigen- and antibody-negative specimens were tested by radioimmunoassay (RIA) for detection of both markers. Compared with the standard tests, the EIA kits for anti-HBs and HBsAg performed well; sensitivity and specificity were 90·3 and 96·0%, respectively, for antibody, and 97·8 and 97·9% respectively for antigen. Substantial disagreement between the EIA and RIA tests for HBsAg was found only for specimens considered weakly reactive by EIA. Few differences were found between three EIA method options for follow-up HBsAg testing of weakly reactive serum specimens; each option contributed about equally to improved test specificity for these 'borderline' specimens. Based on their demonstrated equivalence to the standard RIA tests, we conclude that the EIA kits for anti-HBs and HBsAg detection are suitable for use in hepatitis B control programmes in open populations.

INTRODUCTION

Hepatitis B virus (HBV) infection with its large number of chronic carriers is an important international health problem. In particular there is evidence which strongly supports a causal relationship between hepatitis B antigenaemia and primary hepatocellular carcinoma, particularly in developing countries [1–3]. Control of HBV through immunization has become a recognized public health goal and immunization programmes may require screening large populations for HBV 'markers', i.e. antibody, antigen, or both.

Although radioimmunoassay (RIA) is the standard test method for detecting markers of HBV infection, this method is often impractical for field and regional public health use because of its expense and dependence on complex equipment. In addition, the short shelf-life and transport and disposal of radioactive reagents contribute to the disadvantages of RIA. In contrast, the enzyme immunoassay (EIA) which is recommended by the World Health Organization (WHO) for many public health purposes [4] is frequently available commercially at a lower cost, and uses stable immunoreagents and less complex equipment. In previous comparisons of more limited scope, EIA and RIA methods for detecting serum hepatitis B surface antigen (HBsAg) gave similar results [5–11]. However, EIA has not been evaluated in a large open population with a high prevalence of HBsAg, a common situation where hepatitis programmes are needed most. The hepatitis B immunization project in American Samoa, sponsored by the U.S. Centers for Disease Control (CDC), provided an opportunity to evaluate EIA as a screening method for HBV marker detection in a large open population.

MATERIALS AND METHODS*

Specimen collection and handling

Venous blood specimens were obtained with free and informal consent by either the Vacutainer system with separator tubes (Becton Dickinson & Co., Paramus, NJ), or disposable syringes. These latter specimens were immediately dispensed into Vacutainer separator tubes. Specimens drawn in the field were held at ambient temperature (20–26 °C) for no more than 6 h before centrifugation. Because of the large number of specimens, at times the laboratory was unable to separate serum immediately. In such cases, the specimens were centrifuged at 2000 rev./min for 10 min and stored at 4 °C until the EIA assays were completed by the American Samoa laboratory, usually within 2 weeks, and then frozen at −20 °C before shipment to CDC where all RIA assays were performed. Although RIA was performed on frozen and thawed specimens, studies have shown no decrease in sensitivity/specificity and thus do not invalidate this comparison of assay formats.

Equipment and supplies

All reagents and materials were supplied as commercially prepared kits (Abbott Laboratories, North Chicago, Il), and the enzyme reaction was measured in a Quantum II spectrophotometric analyser (Abbott Laboratories).

Procedures

EIA and RIA procedures were performed in accordance with manufacturer's instructions. As noted below, the algorithm for follow-up by EIA for HBsAg-positive specimens was altered in some cases. In addition to the quality control measures specified by the manufacturer, positive control serum specimens from persons in American Samoa were included at the beginning and end of each set of approximately 300 specimens.

* Use of tradenames is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

Antibody to hepatitis B surface antigen (anti-HBs)

Anti-HBs was determined with the Ausab EIA (Abbott Laboratories), a solid-phase 'sandwich' EIA that uses biotinylated human HBsAg and avidin–horseradish peroxidase conjugate. Of the manufacturer's two time and temperature options, incubation of patient serum and antigen-coated beads, for 16–20 h at 20–22 °C was chosen. Two positive and three negative control serum specimens were included in each set of approximately 300 specimens, and the absorbance of all samples was read at 492 nm. In accordance with the manufacturer's instructions, a negative 'cut-off value' was defined as 0·05 plus the mean absorbance; values < 90% of the cut-off value were considered negative, and those > 110% of the cut off value were considered positive. Specimens with intermediate values were considered equivocal and retested. Final interpretation was based on the second test result.

HBsAg

At the beginning of the study in December 1985, the Auszyme IIa test kits (Abbott Laboratories) were used for detection of HBsAg. These kits included an HBsAg hyperimmune guinea-pig polyclonal antiserum as the source of capture antibody and an HBsAg hyperimmune goat polyclonal antibody conjugated with horseradish peroxidase as the source of detecting antibody. Of the manufacturer's three procedural options for incubation time and temperature, incubation at 20 °C for 12–20 h was selected. In February 1986, the Auszyme IIa test kits were replaced by the Auszyme Monoclonal EIA kit (Abbott Laboratories), which uses mouse monoclonal antibodies both as antibody capture reagent and, after reaction with horseradish peroxidase conjugate, as antibody detection reagent. Four reagent lots were used in sequence as they became available while undergoing developmental changes. The one-step procedure involved simultaneous incubation of patient's serum with both antibody-coated beads and antibody conjugate. The negative cut-off value was determined in the same way for the monoclonal/polyclonal tests. Initially, specimens with absorbance values less than 0·500 were retested and final interpretation was based on the repeat value.

HBsAg confirmatory assay

The manufacturer recommends that a neutralization or confirmatory assay be performed to confirm specimens reactive by the Auszyme monoclonal test. In this, an aliquot of the suspected HBsAg-positive serum was reacted with a human anti-HBs-containing serum for 15 min at room temperature (20 °C) before testing by procedures identical to those of the initial monoclonal test. Aliquots of both the pre-incubated serum and the unreacted serum were assayed in parallel and absorbance values compared. Values for the pre-incubated aliquots at least 50% lower than those of the untreated aliquots were considered indicative of the presence of HBsAg.

In some instances, a high titre of HBsAg in undiluted serum was not fully neutralized by the anti-HBs confirmatory reagent. These specimens required a dilution of 1:25 or 1:500 with negative control reagent prior to testing by the

confirmatory assay in order to exhibit the 50% difference between pre-incubated and untreated aliquots.

HBsAg and anti-HBs RIA

The Ausria IIa test for HBsAg and the Ausab RIA1 test for anti-HBs (Abbot Laboratories) were used at the Hepatitis Branch, Division of Viral Diseases, CDC, Atlanta to retest all specimens forwarded by the laboratory in Samoa.

Sampling protocol

The sample universe was the entire population of American Samoa, estimated at 36000 in 1985 [12]. Attempts were made to study all 36000 persons although children aged 0–5 years were incompletely sampled due to difficulties in obtaining blood specimens. However, a preliminary sero-survey had revealed that approximately 85% of individuals in this age group were susceptible to HBV and a decision was made to forgo testing and to vaccinate routinely this age group. Ultimately, serum samples from approximately 28000 persons obtained between December 1985 and August 1987 were tested for HBV markers (78% sample of the estimated population). The subsample examined in this study consists of 19184 specimens screened between December 1985 and May 1987, the period during which co-author J.A.M. directed the Samoa laboratory work. Overall, 53% of the total estimated population of American Samoa, or 68% of persons cooperating in the entire sample, were included in this study. Specimens were tested in several sequences of EIA and RIA tests (Fig. 1, Table 1). In the interest of economy, the anti-HBs EIA was performed first on all 19184 specimens as a 'pre-screen' (i.e. the initial screening for anti-HBs). This identified immune (anti-HBs positive) individuals and, based on the assumption that they lacked HBsAg, eliminated them from further testing (7200 specimens). Anti-HBc was not used for this screening because a prior serosurvey in this population indicated that, particularly in older age groups, 6·8% lacked anti-HBc although anti-HBs was present, which suggested that anti-HBc became undetectable with time in persons who were naturally infected and who were not vaccinated.

The remaining 11984 antibody-negative specimens were then screened for HBsAg by EIA; 9865 of them (82·3%) lacked detectable antigen. The remaining 2119 specimens (17·1% of the pre-screened antibody-negative specimens) were classified as either strongly or weakly positive. Although the manufacturer of the EIA test kit recommends retest of all HBsAg EIA reactive specimens, regardless of absorbance values, in this study strongly positive specimens (absorbance values > 2) were accepted as HBsAg positive and were not retested by EIA (1526 specimens, 12·7% of the pre-screened total). Additional testing of repeatedly-reactive specimens with the EIA confirmatory assay is then recommended.

All but 83 of the 532 weakly EIA positive specimens (absorbance values < 2) received some form of follow-up test (Fig. 1, Table 1). Repeat EIA for HBsAg was performed on 178 specimens and 73 of these were additionally tested by the EIA Confirmatory Assay. The remaining 271 weakly EIA positive specimens were followed up with the EIA confirmatory assay alone.

Subsequently, all specimens considered positive for HBsAg by EIA were forwarded to CDC. All specimens were tested for HBsAg by RIA, including the

Table 1. *Chronological sequence of laboratory tests to detect HBsAg and anti-HBs, serum specimens from 19184 persons, American Samoa, December 1985–May 1987*

Chronological test sequence				Number specimens tested	Number (%) sampled by RIA for anti-HBs or HBsAg
Step 1 EIA Anti-HBs	Step 2 EIA HBsAg	Step 3 EIA HBsAg	Step 4 EIA HBsAg confirm		
Positive	—	—	—	7200	720 (10)
Negative	Negative	—	—	9850	985 (10)
Negative	Negative	—	—	15	0* (0)
Negative	Highly positive	No	No	1526	1526 (100)
Negative	Low positive	Yes	Yes	73	73 (100)
Negative	Low positive	Yes	No	105	105 (100)
Negative	Low positive	No	Yes	271	271 (100)
Negative	Low positive	No	No	83	83 (100)
Negative	High and low positive	No	No	61	0† (0)
			Total	19184	3763

* Insufficient quantities of serum were available for further testing of 15 of 9865 specimens negative for anti-HBs and negative for HBsAg by EIA.

† Insufficient quantities of serum were available for further testing of 61 or 2119 specimens negative for anti-HBs and positive for HBsAg by EIA.

83 specimens whose HBsAg screen positivity had inadvertently not been confirmed by an additional EIA. Of the 2119 specimens initially positive for HBsAg by EIA, 61 had insufficient volume for testing by RIA. Likewise, 15 of the 9865 specimens negative for HBsAg by EIA had insufficient volume for RIA testing (Fig. 1, Table 1).

In addition, 10% random samples of each of the following two specimen groups were tested by CDC for both HBsAg and anti-HBs by RIA: (1) the 9865 specimens which were found negative by both the anti-HBs pre-screen and the HBsAg screen by EIA: and (2) the 7200 specimens which were not tested for HBsAg by EIA due to the detection of anti-HBs.

Statistical methods

For the EIA anti-HBs pre-screen and HBsAg tests, unadjusted validity measures of sensitivity and specificity were calculated by comparing the final EIA results with the results of the RIA tests, using only those specimens tested by both EIA and RIA (anti-HBs: $N = 1705$; HBsAg: $N = 3043$; Fig. 1).

Adjusted validity parameters for both the antigen and antibody tests were also calculated to correct for the following three factors: (1) the 10% random sampling of the specimens that were EIA anti-HBs positive on pre-screen, and also those that were both negative on EIA anti-HBS pre-screen and negative on initial

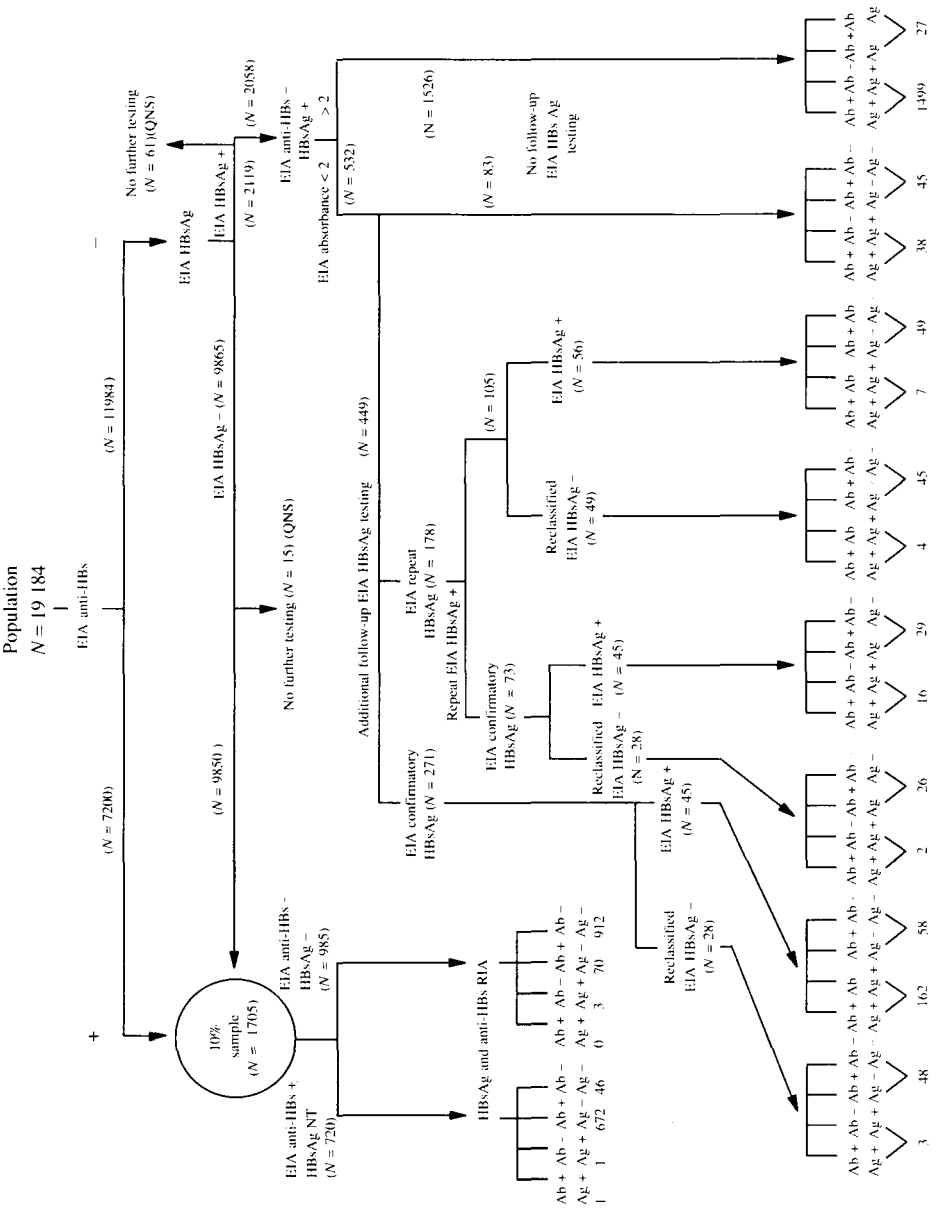

Fig. 1. Schematic diagram of testing procedures.

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Table 2. *Unadjusted and adjusted validity parameters, expressed as percentage, of 19184 EIA tests for anti-HBs and HBsAg, American Samoa, 1985–7*

	Anti-HBs (%)		HBsAg (%)	
Parameter	Unadjusted	Adjusted	Unadjusted	Adjusted
Sensitivity	90·6	90·3	99·3	97·8
Specificity	95·1	96·0	84·1	97·9
False positivity	4·9	4·0	15·9	3·2
False negativity	9·4	9·7	0·7	1·7
Positive predictive value	93·5	93·5	89·2	89·1
Negative predictive value	92·9	94·0	98·0	99·6

HBsAg screen (Fig. 1); (2) the lack of RIA anti-HBs testing in the specimens that were initially both EIA anti-HBs negative and EIA HBsAg positive ($N = 2058$; Fig. 1); and (3) the lack of RIA or further EIA tests for those 61 specimens which were negative for anti-HBs and positive for HBsAg, as well as the 15 specimens negative for both anti-HBs and HBsAg by EIA (Fig. 1, Table 2).

In making these adjustments, we assumed that: (1) the false positivity rates and false negativity rates for the 10% sampled and 90% unsampled specimens (EIA anti-HBs positive and EIA anti-HBs negative/EIA HBsAg negative) were the same; (2) the EIA anti-HBs false negativity rate, determined solely from those 985 specimens tested for both antigen and antibody by both RIA and EIA (Fig. 1), was the same for the 327 anti-HBs-untested specimens ultimately shown to be HBsAg negative by RIA (Fig. 1); and (3) the rates of EIA HBsAg false positivity and anti-HBs false negativity for those specimens without RIA antigen tests, or without RIA antibody tests ($N = 61$ and $N = 15$, respectively, Fig. 1), were the same as those in which such testing had been done. The resultant estimated numbers were then utilized for the adjustment validity parameter calculations (Table 1).

SPSSx (SPSS Inc., Chicago, Illinois) software was used on the VAX 11/750 (Digital Equipment Corporation, Maynard, MA) for statistical analyses. Fisher's Exact test was used for comparison of validity parameters.

RESULTS

Detection of anti-HBs

The Abbott Ausab EIA pre-screen test detected anti-HBs in 673 of the 743 specimens determined to be anti-HBs positive by RIA (unadjusted sensitivity 90·6%). The EIA anti-HBs assay failed to detect antibody in 915 of the 962 specimens determined to be without anti-HBs by the RIA assay (unadjusted specificity 95·1%). When the adjustment factors delineated previously were utilized, only slight changes in the validity parameters resulted (Table 2). The adjusted specificity of the EIA anti-HBs test in the total population subsample was 96·0% and the false positivity 4·0%. In the entire population subsample, the predictive value of a positive test, 'positive predictive value', was 93·5% and the negative predictive value 94·0% (Table 2).

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Table 3. *Comparison of validity parameters, expressed as percentage, of three different EIA follow-up methods for 449 anti-HBs negative sera with absorbance values < 2 on initial EIA HBsAg screening, American Samoa, 1985–7*

Parameter	Follow-up method*		
	Repeat EIA HBsAg and confirmatory	Repeat EIA HBsAg alone	EIA confirmatory assay alone
Sensitivity	89·9 (18)	63·6 (11)	98·2 (165)
Specificity	47·3 (55)	47·9 (94)	45·3 (106)
False positivity	52·7 (55)	52·1 (94)	54·7 (106)
False negativity	11·1 (18)	36·4 (11)	1·8 (165)
Positive predictive value	35·6 (45)	12·5† (56)	73·6‡ (220)
Negative predictive value	11·1 (28)	91·8 (49)	94·1 (51)
Sera (*N*)...	73	105	271

* ( ) Number of specimens from which the validity parameter was determined.

† $P < 0{\cdot}05$, repeat HBsAg alone, compared with both the repeat EIA HBsAg and the confirmatory assay.

‡ $P < 0{\cdot}001$, EIA confirmatory assay alone, compared with both the repeat EIA HBsAg and the confirmatory assay.

Detection of HBsAg

Of the 1734 specimens which ultimately had HBsAg detected by RIA, 1722 were initially detected by the Abbott Auszyme II and Auszyme Monoclonal tests (sensitivity 99·3%). Using the adjusted numbers, 1773 of the 1812 positive specimens had been correctly identified by the EIA screen, for an 'adjusted' EIA test sensitivity of 97·8% (Table 2).

The unadjusted specificity of the EIA HBsAg test was 84·1%, determined by comparing the 1101 specimens lacking HBsAg by EIA with the 1309 specimens without detectable HBsAg by RIA. Comparing the adjusted figures, 9954 of the 10172 specimens negative for HBsAg by RIA had been similarly negative by EIA, giving an adjusted specificity of 97·9%. The lower unadjusted sensitivity reflects the lack of weighting which is necessary to account for the 10% sampling process. When adjustment equalizes the influence of this sample, a more meaningful sensitivity results. The positive and negative predictive values of the EIA HBsAg tests, adjusted for sampling, were 89·1 and 99·6%, respectively (Table 2).

Follow-up methods for confirmation of weakly reactive HBsAg specimens

Calculation of validity parameters (sensitivity, specificity, positive and negative predictive values) for the method recommended by the manufacturer for follow-up of specimens weakly reactive for HBsAg (repeat HBsAg EIA and EIA confirmatory assay) yielded a sensitivity of 88·9% and a specificity of 47·3% (Table 3). Validity parameters for follow-up testing of weakly positive specimens by either repeat HBsAg EIA alone or EIA confirmatory assay alone yielded results comparable to those for the recommended method, in which the two

Table 4. *Comparison of validity parameters, expressed in percentage, of EIA HBsAg tests, by detecting antibody reagent source and lot number, for 449 weakly-reactive and 1526 strongly-reactive sera, American Samoa, 1985–7**

Parameter	Polyclonal antibody reagent Lot number 82192HR	Monoclonal antibody reagent Lot number 1 85578HR	Lot number 2 87946HR	Lot number 3 90434HR	Lot number 4 93886HR	All lot numbers	All lot numbers and reagent sources
Sensitivity	100·0 (172)	98·9 (452)	98·6 (284)	100·0 (231)	100·0 (221)	99·4 (1558)	99·4 (1731)
Specificity	20·0 (20)	28·2 (156)	47·7 (44)†	60·0 (35)†‡	51·3 (39)†‡	37·5 (307)	36·4 (327)
False positivity	80·0 (20)	71·8 (156)	52·3 (44)†	40·0 (35)†‡	48·7 (39)†‡	62·5 (307)	63·6 (327)
False negativity	0·0 (172)	1·1 (452)	1·4 (284)	0·0 (231)	0·0 (221)	0·6 (1558)	0·6 (1731)
Positive predictive value	91·4 (188)	80·0 (559)‡	92·4 (303)	94·3 (245)†	92·1 (240)	89·0 (1740)	89·2 (1929)
Negative predictive value	100·0 (4)	89·8 (49)	84·0 (25)	100·0 (21)	100·0 (20)	92·0 (125)	92·2 (129)

* Number of specimens for which the validity parameter was determined.
† Significant difference between monoclonal reagent lot number 1 (85578HR) and other monoclonal reagent lot numbers, ($P < 0·05$).
‡ Significant difference between polyclonal reagent and monoclonal reagent ($P < 0·05$).

procedures were performed in sequence. Sensitivities ranged from 89 to 100% and specificities from 44 to 47% (Table 3). Validity parameters for follow-up testing of weakly positive specimens by either repeat HBsAg EIA alone or EIA confirmatory alone yielded results comparable to those for the recommended method, in which the two procedures were performed in sequence. Sensitivities ranged from 64 to 98% and specificities from 45 to 48% (Table 3). The only significant differences between the three procedures were in positive predictive value (Table 3). Although the sensitivity of the repeat HBsAg alone was significantly different than that of the confirmatory assay alone, neither was significantly different from the recommended procedure.

Comparisons between different HBsAg EIA reagents

Validity parameters determined on all specimens reactive for HBsAg by EIA by the source and lot number of antibody reagent used in the HBsAg EIA tests revealed that monoclonal and polyclonal lots were generally comparable (Table 4). Differences in specificity between the specimen groups associated with the individual lots were noted. Both polyclonal antibody reagent lot 82192HR and one monoclonal reagent (lot 85578HR), which had similar parameter values, had significantly different specificity and false positivity values from those of the three other monoclonal reagents ($P < 0{\cdot}05$, Fisher's Exact test). Differences were found in positive predictive value between this monoclonal reagent and the polyclonal reagent, as well as between one other monoclonal reagent (lot 90434HR) and this monoclonal reagent ($P < 0{\cdot}05$, Fisher's Exact test). No other significant differences were found between reagent lots.

DISCUSSION

Under conditions of mass screening in an open population with high prevalence of both anti-HBs and HBsAg, the commercially available EIA kit test compared favourably with the standard RIA procedure for detection of HBsAg. The resultant high sensitivity (97·8%) and specificity (97·9%) may make the simpler and cheaper EIA method of choice for many situations.

Disagreement between the EIA and RIA tests for HBsAg detection occurred only for serum specimens that were weakly reactive for HBsAg on the initial EIA screen. Follow-up tests on these specimens by any of three possible EIA methods (EIA repeat, EIA confirmatory assay, or both tests in sequence) resulted in test specificities between 43 and 48%. However, it was not possible to determine from these data whether the low specificity associated with the weakly reactive specimens reflects poor performance of one or both of the tests in detecting very low levels of HBsAg. Further study of subjects with weak reactions over long periods of time may clarify this issue. Even in this population of high HBsAg prevalence, however, weakly reactive specimens represent only about one-fourth of the positive specimens and merely 3·0% of the total specimens tested, thus having minimal impact on the screening programme.

The only significant differences between the three EIA HBsAg follow-up methods for weakly reactive EIA specimens were in the positive predictive values (Table 3), probably indicating unequal selection of specimens in each category.

Comparison of the positive predictive value between the three follow-up methods is of limited value in this situation because only a subsample of the population is being evaluated and this subsample consists solely of specimens which produced at least one positive EIA HBsAg result. The data thus suggest that under cost-containing or time-limiting constraints, either repeat HBsAg EIA alone, or EIA HBsAg confirmatory assay alone is an acceptable method that yields equivalent results.

Evaluation of the validity parameters for kits with polyclonal and monoclonal antibody reagents showed generally comparable results, suggesting that the different reagents did not greatly affect the ability of test kits to identify HBsAg positive and negative sera. Minimal secular changes in test validity, as shown by the chronological numbering of test reagent lots (Table 4), may merely reflect the continuing development of this first-generation EIA monoclonal test for HBsAg.

Taken together, the EIA anti-HBs pre-screen coupled with the HBsAg screen successfully detected persons with HBsAg. The low sensitivity of the EIA anti-HBs test (90·3%) and the low specificity of the EIA HBsAg follow-up tests of weakly reactive sera resulted in a compromise: a few false-positive HBsAg results were accepted to avoid missing persons who were truly antigenaemic. Thus the combination of the two tests in screening for HBsAg was effective in detecting antigenaemic persons at the expense of initially misclassifying a few individuals who were not antigenaemic.

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