SCREENING TEST ACCURACY AMONG POTENTIAL BLOOD DONORS OF HBSAG, ANTI-HBC AND ANTI-HCV TO DETECT HEPATITIS B AND C VIRUS INFECTION IN RURAL CAMBODIA AND VIETNAM

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Abstract. The aim of this study was to determine the accuracy of rapid tests for HBsAg, anti-HBc and anti-HCV in rural Cambodia and Vietnam to detect hepatitis B and C. In a cross-sectional epidemiological study of two populations of 1,200 potential blood donors in rural Cambodia and Vietnam the prevalence rates of HBsAg, anti-HBc and anti-HCV as established by enzyme immunoassay (EIA) tests were compared to rapid test outcomes. The EIA reference test results were validated by Architect Chemiluminescent Microparticle Immunoassay (CMIA) technique. The actual rapid test demonstrated high specificity for all three test categories as claimed by the manufacturer. The test sensitivity observed was significantly lower than that claimed by the manufacturer: 86.5% for HBsAg, 86.6% for anti-HBc, and 76.4% for anti-HCV. There were large and significant variations in test performance between the two countries, especially for HBsAg detection. The low sensitivity of the actual rapid tests for HBsAg, anti-HBc and anti-HCV make them useless for blood donor screening in rural Southeast Asia. Rapid tests may be useful screening tools in blood transfusion services in low-resource settings, but tests should be carefully validated locally before being used for screening purposes since test performance varies by location.

Key words: hepatitis B surface antigen (HBsAg), hepatitis B core antibody (anti-HBc), hepatitis C Virus (HCV), hepatitis C (anti-HCV), rapid test, blood antibodies donor screening, Vietnam, Cambodia

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INTRODUCTION

Safe blood transfusions are difficult to achieve in developing countries where resources are limited and blood-transmitted

diseases are endemic. Among transfusion transmitted infections, HBV is the most common with risk estimates at 1:60,000 in countries where the prevalence is low. Where hepatitis B is endemic, transmission rates are probably much higher and occur in part due to improper testing (Wang *et al*, 2002). Testing for HBsAg is in place in most low-income countries. However, transmission can still occur during the initial window-period of an acute infection, or during late stages where virus is still present (HBV-DNA positive) though HBsAg is negative, so-called occult hepatitis B infection (OBI) (Liu *et al*, 2006; Bhattacharya *et al*, 2007; Hollinger, 2008; Niederhauser *et al*, 2008). A recent major epidemiological multi-center study of potential blood donors in rural Cambodia and Vietnam reports prevalence rates of HBsAg-negative, anti-HBc-positive cases in the study population as high as 42% and 51%. Based on estimates from other studies, as many as 10% of HBsAg-negative donors in these areas may be potential transmitters of HBV infection by blood donation (Ol *et al*, 2009; Viet *et al*, unpublished data). Accurate detection of HBV and HCV carriers and anti-HBc-positive donors is an urgent issue in order to set standards for safe blood transfusion where HBV infections are endemic.

Rapid tests may yield false test outcomes due to the prozone effect, especially during the initial phase after infection when the viral load is high and there are high antigen concentrations (Seidl and Trautmann, 1981; Van de Perre *et al*, 1988; Pujol, 1993), and due to genotype variations that may influence test sensitivity (Mizuochi *et al*, 2005; Kuiken *et al*, 2007 Huy *et al*, 2008). Serological tests, such as enzyme immunoassays (EIA), have high accuracy in detection of serological markers, such as HBsAg, anti-HBc and anti-HCV. However, the tests are expensive, require complex instrumentation, and are not feasible in rural remote district hospitals in low-income countries. Advances in diagnostic technology have resulted in rapid tests for identification of serological markers. However, the accuracy of these rapid tests as claimed by the manufacturers is normally based on seroconversion test panels which do not necessarily reflect the antibody or antigen spectrum in the population studied. It is possible that test accurate on pre-arranged panels may yield falsely high performance indicators.

The aim of the study was to determine the accuracy of one rapid test system for detection of HBsAg, anti-HBc and anti-HCV in two study populations in rural Southeast Asia.

MATERIALS AND METHODS

This was a cross-sectional epidemiological study of potential blood donors in rural Cambodia and Vietnam. The study was carried out in February-June 2007 in Battambang and Pailin Provinces, Kingdom of Cambodia and Quang Tri Province, Vietnam.

Study subjects

The study population consisted of 2,400 female and male potential blood donors from rural Cambodia and Vietnam, 1,200 from each country. The mean age of the study population was 33.4 years (SD 9.5); 59% were females. The ages did not differ significantly between the subjects from the 2 study countries, but the rate of female participants was slightly higher in Vietnam (95% CI diff: 0.5%-8.4%). Prior to blood sampling, the villagers were informed by local health authorities the study would be done in order to establish

safe blood service for the population, that participation was voluntary and free of charge; that all participants would be informed of the test outcome and they would obtain medical advice and counseling. Voluntary participants were registered and a consent form was signed before sampling. For inclusion, the study participants should be living permanently in the study area, and should be 18-55 years old. Participants previously vaccinated for HBV were excluded from the study.

Sampling

One blood sample was taken from each subjects by trained laboratory technicians, set aside to coagulated for 30 minutes, then centrifuged and cooled to 4ºC in portable cooling boxes. The serum samples were then taken for analysis at Battambang Blood Transfusion Center in Cambodia or Quang Tri Provincial Preventive Medicine Center, Vietnam and analyzed within three days after sampling. All samples were processed according to manufacturers instructions and included in the study sample.

Rapid tests

The rapid tests were studied since they were already in use for blood donor screening in some parts of Cambodia. Three qualitative chromatographic immunoassays for HBsAg, anti-HBc, anti-HCV were studied: ACON® HBsAg one step ultra, no. IHBsg-U302; ACON® anti-HBc one step, no. IHBcb-302; and ACON® anti-HCV one step, no. IHC-302 (Acon Laboratories, San Diego, CA). The HBsAg test utilizes a combination of monoclonal and polyclonal antibodies to selectively detect elevated levels of HBsAg in serum and plasma. The test detection limit is claimed to be 0.5 ng/ml for HBsAg. The HBsAg test is claimed to detect both ad and ay subtypes at concentrations of 0 to 300 ng/ml. The anti-HBc test utilizes a combination of monoclonal antibodies and antigen to selectively detect elevated levels of anti-HBc in serum or plasma.

The anti-HCV test utilizes a combination of protein A coated particles and recombinant HCV proteins to selectively detect antibody to HCV in plasma or serum. The recombinant antigen used in the test was encoded by genes for both structural and non-structural proteins. All three tests had a procedural internal control (Acon laboratories, n.d.). The epidemiological test properties (sensitivity and specificity) given by the producer are shown in Table 1.

The rapid tests were carried out according to the manufacturers' instructions. The rapid test outcomes were compared to the test properties claimed by the manufacturer. Agreement analysis was undertaken to compare the overall rapid test outcome with enzyme immunoassay (EIA) as a reference test.

Reference tests

The EIA reference test (Monolisa EIA Assay® BioRad; Monolisa® HBsAg ultra, no. 72348; Monolisa® anti-HBc plus, no. 72316; Monolisa® anti-HCV plus, no. 72318 (BioRad Diagnostics, Pleasanton, CA) was carried out at either Battambang Blood Transfusion Center in Cambodia or the Quang Tri Provincial Preventive Medicine Center in Vietnam. The EIA test measures a numerical ratio (S/CO) for classification of test-positives and test-negatives, increasing ratios indicating higher concentrations of detected antibodies or antigens. Ratios lower than 1.0 were classified as a negative result; ratios higher or equal to 1.0 were classified as a positive result; units with a ratio in the range of 0.9-1.0 were classified as equivocal and re-analyzed (Murray *et al*, 2007; www3.bio-rad.com). The lower limit of sensitivity of the

Table 1
Test properties as claimed by the producers: sensitivity and specificity estimates given by 95% confidence intervals.

	EIA test			Rapid test		
	HBsAg	anti-HBc	anti-HCV	HBsAg	anti-HBc	anti-HCV
Sensitivity (%)	100 (99.1-100)	99.53 (98.3-99.9)	100 (99.3-100)	>99.0 (97.6-100)	96.3 (94.1-97.8)	96.8 (91.0-99.3)
Specificity (%)	99.94 (99.9-100)	99.9 (99.8-100)	99.8 (99.7-99.9)	>99.0 (97.6-100)	96.8 (91.9-99.1)	99.0 (98.4-99.4)

Monolisa HBsAg test was 0.05 IU/ml. The EIA test properties are given in Table 1.

The EIA test outcome was validated with the Automated Chemiluminescent Microparticle Immunoassay Technique (CMIA, Abbott, Wiesbaden, Germany). A subset of 640 serum samples (n=240 for each category, HBsAg, anti-HBc, and HCV) was randomly selected for validation at the Department of Microbiology, University Hospital of North Norway. The subset sample size (n = 240) was estimated in order to detect test indicator differences of more than 5% with 95% confidence, the subset being selected to get at a balance of 2/3 assumed test-positive units versus 1/3 assumed test-negative units (Fig 1). With the CMIA testing of HBsAg, samples with values less than 0.05 IU/ml were considered negative and those with values greater than or equal to 0.05 IU/ml were considered positive. CMIA analysis of anti-HBc and anti-HCV was based on the ratio of the signal to the cut-off value (S/CO). A ratio less than 1.00 was classified as negative, and a ratio greater than 1.00 was classified as positive. Ratios in the range of 0.90-1.00 were classified as equivocal and reanalyzed (Murray *et al*, 2007; Abbott Diagnostics., n.d.). CMIA validation was performed blinded and demonstrated a high agreement between the EIA and CMIA test outcomes, with kappa values higher than 0.8 for HBsAg, anti-HBc, and anti-HCV detection. Based on these results, the EIA test outcomes for HBsAg, anti-HBc, and anti-HCV in the total study population (N = 2,400) were used as a reference for evaluation of rapid test accuracy in the actual study.

Data analysis

The data were processed in a relations database and later merged into one joint database for statistical analysis (JMP 6.0.2. SAS Institute). After descriptive analyses of the data using graphical and tabular analyses, further test evaluation was done comparing the rapid tests to the EIA reference test: sensitivity, specificity, false positive / negative proportions and the kappa coefficient of agreement were calculated. Continuously distributed variables are expressed as mean values with 95% confidence intervals constructed by the Student procedure. Categorical variables are presented in contingency tables with 95% confidence intervals for two-tailed comparison between the groups (Agresti, 2002). Kappa (κ) analysis was used to express agreement between test methods; κ-values of 0.4-0.6 were classified as having acceptable agreement, values of 0.6-0.8 as having high agreement, and values of 0.8-1 as having very high agreement (Altman, 1999).

Table 2
Rapid test (n = 2,400) versus EIA reference test at the two study locations, estimates given by 95% confidence intervals.

	HBs Ag		Anti-HBc		Anti-HCV	
	Cambodia	Vietnam	Cambodia	Vietnam	Cambodia	Vietnam
Rapid test prevalence (%)	7.3 (5.9-8.9)	9.5 (7.9-11.3)	53.2 (50.3-56.0)	44.4 (41.6-47.2)	11.5 (9.7-13.3)	-
EIA test prevalence (%)	7.7 (6.2-9.3)	11.4 (9.6-13.2)	58.6 (55.8-61.4)	51.4 (48.8-54.5)	14.7 (12.7-16.7)	-
Sensitivity (%)	93.5 (86.3-97.6)	81.8 (74.3-87.8)	89.3 (87.0-91.6)	83.6 (80.6-86.5)	77.3 (70.4-83.2)	-
Specificity (%)	99.9 (99.5-100)	99.8 (99.3 -100)	98.0 (96.3-99.0)	97.4 (95.8-98.6)	99.8 (99.3-100)	-
False-negative rate (%)	6.5 (2.43-13.7)	18.3 (12.2-25.8)	10.7 (8.4-13.0)	16.5 (13.5-19.4)	22.7 (16.8 -29.6)	-
Kappa	0.96 (0.93-0.99)	0.88 (0.84-0.92)	0.86 (0.83-0.89)	0.81 (0.78-0.84)	0.85 (0.80-0.89)	-

Table 3
Rapid test performance (n = 2,400), compared to EIA reference test, estimates given by 95% confidence intervals.

	HBs Ag	Anti-HBc	Anti-HCV
Rapid test prevalence (%)	8.4 (7.3-9.6)	48.8 (46.8-50.8)	6.0 (5.1-7.1)
EIA test prevalence (%)	9.5 (8.4-10.8)	55.1 (53.1-57.1)	7.4 (6.4-8.5)
Sensitivity (%)	86.5 (82.0-90.9)	86.6 (84.8-88.5)	76.4 (69.5-82.4)
Specificity (%)	99.9 (99.6-100)	97.7 (96.6-98.5)	99.6 (99.2-99.8)
False negative rate (%)	12.4 (8.1-16.7)	13.2 (11.2-14.8)	22.7 (16.8-29.6)
False positive rate (%)	0.14 (0.03-0.4)	2.49 (1.65-3.6)	0.41 (0.19-0.77)
Kappa	0.91 (0.88-0.94)	0.83 (0.81-0.85)	0.83 (0.78-0.88)

Ethics

Patient consent was given after receiving both oral and written information by local health workers and investigators, and medical counselling was based on test outcomes. Patient data was kept confidential. The study was approved in Cambodia by the Cambodian Committee for Research Ethics (ref 023 NECHR, 2/4/2007) and in Vietnam by the Quang Tri Health Service and Quang Tri Provincial People's Committee (2472/QD-UBND, 20/12/2006). Data was processed by permission from the Norwegian Social Science Data Service, Norway (ref no. 13702).

RESULTS

Tables 2 and 3 show the results of the rapid tests and EIA tests for both study countries. Table 2 (divided by country) and Table 3 (all results) demonstrate the per-

Table 4
EIA ratios for groups of rapid test-negatives and -positives, mean values given by 95% confidence intervals.

	HBsAg		Anti-HBc		Anti-HCV	
	Negative	Positive	Negative	Positive	Negative	Positive
Mean ratio (SD)	0.41 (1.8)	38.7 (10.5)	0.57 (0.04)	5.87 (1.53)	0.18 (0.41)	7.35 (2.68)
95% CI	0.33, 0.49	37.2, 40.2	0.50, 0.65	5.79, 5.95	0.17, 0.20	6.91, 7.79
Range	0.0-54.9	0.12-54.5	0.01-7.71	0.06-9.74	0.0-8.88	0.05-12.66

formance of the three rapid tests versus the EIA. There were significant differences in test performance between the two study countries, the sensitivity being significantly lower and the rate of false-negatives significantly higher for HBsAg and anti-HBc in the Vietnamese subsample compared to the Cambodian subsample. For the anti-HCV test, the sensitivity was low in Cambodia; inter-country comparison could not be done due to too few HCV positive serum samples in Vietnam (Table 2). The rapid test sensitivity was significantly lower and the false negative rate significantly higher for the anti-HCV test compared to HBsAg and anti-HBc tests (Table 3).

The rapid test-negative results (compared to EIA) for all three test categories are shown in Table 4 and Fig 2. The agreement between test methods (EIA and rapid tests) was high for all three test categories with kappa values of > 0.8.

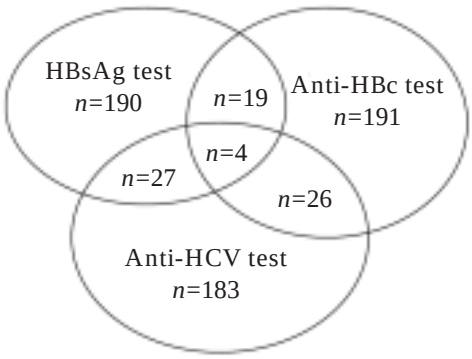

Fig 1–Venn diagram describing the composition and over-lap of the three subsets for CMIA analysis. The numbers within each area indicate the number of study units. Total of 240 samples for each category.

DISCUSSION

The study reveals three main findings. The accuracy of the rapid test for qualitative detection of HBsAg, anti-HBc and anti-HCV in a large study population in Southeast Asia is low. For detection of HCV the false-negative rate was high: one of five anti-HCV positive serum samples being missed. There was a significant difference between the observed and manufacturer claimed test sensitivities for all three test categories. There were significant differences in test sensitivity between two sub-populations: Cambodia and Vietnam.

The poor test performance could be due to methodological flaws affecting crude outcomes as well as the reliability of the findings.

One may question the reliability of the local reference test used for assessment of rapid test performance. During EIA analysis, all study units with a test outcome

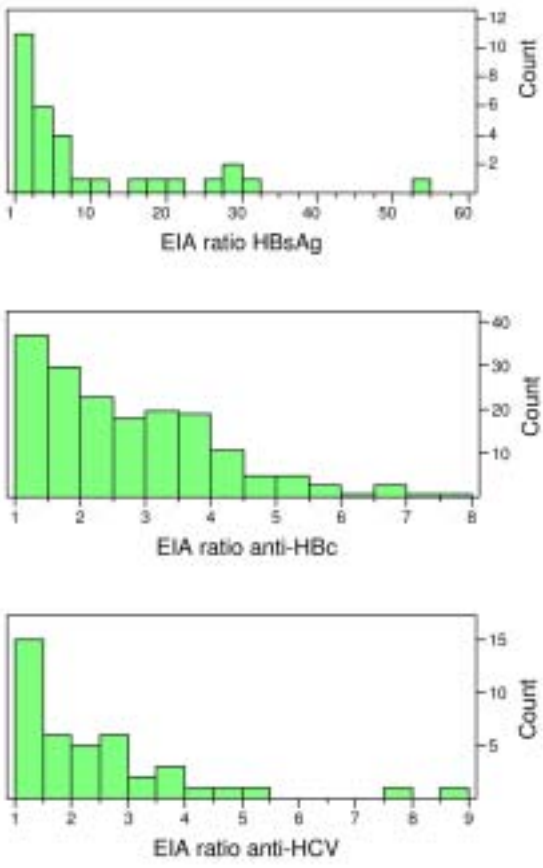

Fig 2–Distribution of EIA ratio values for rapid test false-negative units.

close to the EIA cut-off level were re-analyzed before test results were registered in the database. EIA analysis was validated by CMIA technique in a representative subset of the study population. The validation was performed blindly at a Norwegian medical laboratory of high standard demonstrating high agreement (κ>0.8) between the reference test (EIA) and the CMIA test outcomes for HBsAg, anti-HBc, and anti-HCV. Therefore, there should be no reason to doubt the accuracy of the reference test used in the study.

Failures regarding sampling and processing in the field may have occurred. All procedures were performed strictly according to the protocol under close supervision by the research teams. No accidental events were reported. We can rule out any systematic impact of such technical errors.

The study was undertaken among rural populations where hepatitis is endemic and both antigen and antibody levels may be high. Therefore a prozone effect may explain why some true positive results turned out negative with the rapid tests (Seidl and Trautmann, 1981; Van de Perre *et al*, 1988; Pujol *et al*, 1993). Cross reacting antibodies may also disturb the test results. There may also be test flaws due to genotype variations that may influence test sensitivity. For both HBV and HCV there are genotypes differences between western countries and Southeast Asia. HCV is an extremely variable virus with six different genotypes and more than 70 subtypes; HCV genotype 6 is dominated in Southeast Asia, with large subgenotype differences between countries, *eg* Vietnam: 6a and 6d/e, Cambodia: 6q, Thailand: 6f, 6i/j, 6m and 6n (Kuiken *et al*, 2007; Jia *et al*, 2009; Pybus *et al*, 2009). For HBV the genotypes B and C are the most common in Southeast Asia, while genotypes A and D are dominant in Europe (Norder *et al*, 2004; Schafner, 2005). The low sensitivity and the large difference in sensitivity between countries, especially for HCV, can be related to deficient detection of genotypes and/or subtypes with the tests.

The fact that the rapid test specificity was relative good but the sensitivity was poor, with many of the false negative rapid tests having reference test results far above the cut-off level, by itself indicates the poor rapid test performance in the study population is real (Fig 2).

The results from previous studies of rapid test accuracy in HBV screening are not clear. In a major survey evaluating the accuracy of HBsAg and anti-HCV detection in blood donors in Equador, Grijalva *et al* (2005) reported significantly higher false-negative rates for rapid tests compared to EIA analysis. In a study of HBsAg rapid test performance in a population of high HBV prevalence in Vietnam, Lien *et al* (2000) compared three different rapid tests (the ACON test was not included) and found the tests performed well, but one test had a false-negative rate of 3-4%. The study population in the Vietnamese study was small (n = 117) and the performance estimates consequently imprecise. In our study we found significant differences in test performance for both HBsAg and anti-HBc between the study populations in Cambodia and Vietnam. The findings indicate there are uncontrolled variables affecting test outcomes, such as the prozone effect and genotype variations. The findings show that test accuracy in one study population or in one study country does not grant good performance in another population.

In summary, the sensitivities of the rapid tests for detecting HBsAg, anti-HBc and anti-HCV were low and the false-negative rates were too high to make the tests feasible for blood donor screening in rural Southeast Asia. There were large variations in test performance between the two countries. Rapid tests for HBsAg, anti-HBc, and anti-HCV should be carefully validated locally before being used for blood donor screening. Rapid tests are a reasonable screening tool for blood transfusion services in low-resource settings; further studies of rapid-test accuracy should be carried out on a variety of study populations.

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