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Application of sequential serological testing strategy for detection of Hepatitis B surface antigen (HBsAg) for diagnosing HBV infection

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ABSTRACT

The diagnostic accuracy of any serological test for detection of HBsAg is not 100%. We hypothesized that the sequential testing strategy proven for anti-HIV laboratory diagnosis should also apply to other infectious disease markers like HBsAg. Therefore, we evaluated the diagnostic accuracy of these strategies, I (single test), II (two tests in sequence), III (three tests in sequence) for diagnosis in patients and blood donors and compared it to the confirmatory test for HBsAg (Neutralization Test). Samples were initially tested for HBsAg by A1- Enhanced Chemiluminescent Immuno Assay (ECLIA). Initial reactive (aliquoted donor/patient) samples were reflexly tested by A2- Enzyme Linked Fluorescence Assay (ELFA) and A3- Immuno Chromatography Assay (ICA) assays. Confirmatory neutralization assay was performed on all initial reactive samples. Four strategies (I, II A, II B, and III) that were used in this analysis were; I = A1, IIA = A1 + A2, IIB = A1 + A3, and III = A1 + A2 + A3. The results of all four strategies were compared to Gold Standard (Neutralization Test). A total of, 112, 011 blood samples (75,111 patient samples and 36,900 whole blood donor samples) were initially tested for HBsAg by A-1 (CLIA). Amongst the tested samples, 1,296, 1,188, 1,078, 1,074 samples were found to be reactive by strategy I, IIA, IIB, III respectively. We observed that the PPV (Positive Predictive Value) of Strategy III > Strategy IIB > Strategy IIA > Strategy I. Sequential serological testing strategy comprising of initial sensitive test followed by more specific test increases the diagnostic accuracy of test report as compared to a single test.

Hepatitis B infection is a major public health problem worldwide. According to the World Health Organization (WHO), around 248 million individuals are chronically infected with hepatitis B virus (HBV) in the world; particularly in low- and middle-income countries and four million acute infections are reported every year. Approximately 686,000 people die each year due to complications related to chronic HBV infection (WHO, 2017). Due to limited access to diagnosis and treatment of hepatitis B, in many resource constrained settings, infected individuals are not aware of their status and present with complications in advanced stages of disease. Testing and diagnosis of HBV infection is important for prevention and treatment of hepatitis. The most commonly performed diagnostic test to detect Hepatitis B infection is HBs Ag.

Different methods for detection of HBsAg are available since the demonstration of its relation toviral hepatitis (Blumberg, 2000). Routinely, serum HBsAg can be detected by enzyme linked immunosorbent assay (ELISA), immunochromatographic rapid diagnostic test, electrochemiluminescent immunoassay, and chemiluminescent microparticle immunoassay (Amini et al., 2017; Randrianirina et al., 2008). Since no single test is 100% sensitive and specific, sequential testing strategies comprising of two or more tests to improve diagnostic accuracy have been reported. One of these reports from WHO has defined and classified the testing strategies for HIV into three strategies, namely I, II and III [WHO, HIV Guidelines]. While strategy I may be adequate for blood screening, it is sub-optimal for making diagnosis in a patient or recalling a blood donor for post-donation counselling and follow-up.

We hypothesized that the sequential testing strategy proven for anti-HIV laboratory diagnosis is applicable to other infectious disease markers such as HBsAg. Therefore, we evaluated the diagnostic accuracy of these strategies, I (single test), II (two tests in sequence), III (three tests in sequence) for diagnosis in patients and blood donors and compared it to the confirmatory test for HBsAg, the neutralization test). Several laboratories continue to use strategy I, especially in blood screening. However, when it comes to disease surveillance and diagnosis, laboratories use two or three tests.

This study was a prospective analysis of test results by different serological assays used for detection of HBsAg in patients and blood donors, performed from March 2016 to July 2017 in the department of

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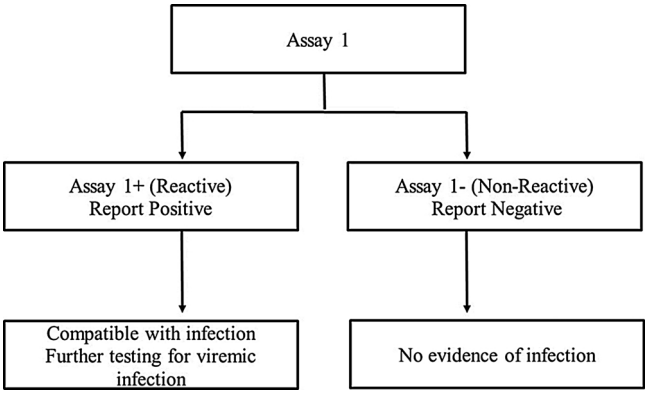

Fig. 1. Testing Strategy I decision tree. This testing strategy uses A1(ECLIA) to screen blood samples.

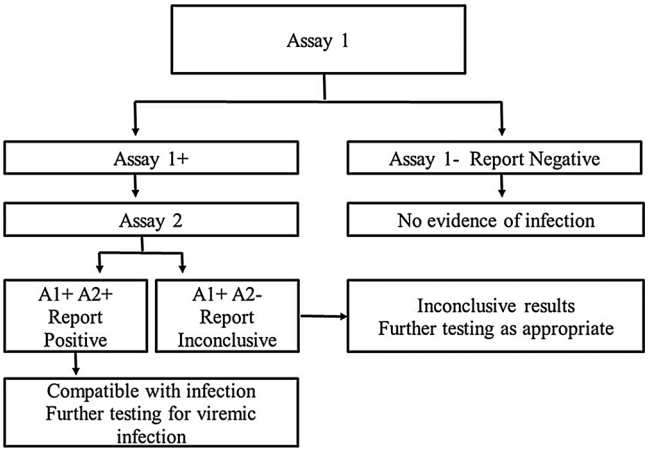

Fig. 2. Testing Strategy II decision tree. This testing strategy uses A1(ECLIA) and A2(ELFA/RDT) to screen blood samples.

Transfusion Medicine of a large tertiary care hospital in north India.

The four testing strategies (I, II A, II B, and III) used in this study were adapted from HIV testing algorithms suggested by WHO as shown in Figs. 1–3. Strategy I uses assay A1, strategy II A uses assays A1 and A2, strategy II B uses A1 and A3, and Strategy III uses A1, A2 and A3. The assays used are detailed bellow:

A1. Enhanced chemiluminescence immunoassay (ECLIA). This method was performed using the VITROS HBsAg ES reagent pack and VITROS immunodiagnostic products HBs Ag calibrator on the VITROS ECIQ and VITROS 3600 immunodiagnostic system. This method is based on immunometric assay. It involves the simultaneous reaction of HBsAg present in the sample with biotinylated antibodies (mouse anti-HBs) and horseradish peroxidise (HRP)-labelled antibody conjugates (anti mouse anti-HBs). Antigen-antibody complexes are captured by streptavidin on the wells. The unbound antibody-enzyme labels are removed by washing. The bound HRP conjugate is measured by a luminescent reaction by adding a signal reagent which contains luminogenic substrate (luminol and peracid salt) and an electron transfer reagent. The HRP catalyses the oxidation of the luminol derivative, producing light. The light signals are measured by the luminometer in the system. The related light unit (RLU) of the reaction is proportional to the concentration of HBsAg present in the specimen.

A2. Enzyme linked fluorescence immunoassay (ELFA). This method is performed in the automated VIDAS system (BioMerieux SA, France). The solid phase receptacle (SPR®) serves as the solid phase as well as the pipetting device. In the initial step, antigen present binds simultaneously to monoclonal antibody of SPR and antibody conjugated to biotin. Unbound antigen is washed away. Bound antigen is conjugated with alkaline phosphatase. After further washing, during the detection step, the substrate (4-methyl-umbelliferyl phosphatase) is catalyzed by the conjugate enzyme into a fluorescent product (4-methyl-umbilliferone) that is measured at 450 nm. The intensity of the fluorescence is proportional to the concentration of antigen present in the sample. At the end of assay, results are analyzed automatically by the instrument and expressed as an index calculated using a standard.

A3. Immunochromatographic Rapid Diagnostic Test (RDT). This is a one-step rapid visual test for the qualitative detection of HBsAg in human serum (Hepacard, J. Mitra and Company, India). It uses monoclonal antibodies conjugated to colloidal gold and polyclonal antibodies immobilized on a nitrocellulose strip in a thin line. The test sample flows laterally through an absorbent pad where it mixes with the signal reagent. HBsAg binds with the colloidal gold-antibody conjugate forming an antigen-antibody colloidal gold complex. This complex is trapped by the immobilized antibody in the test line (T), forming an antibody-antigen-antibody gold complex resulting in a pink band.

Gold standard assay. Neutralization test (NT). It was performed using VITROS HBsAg ES Confirmatory Kit (Ortho Clinical Diagnostics, UK)

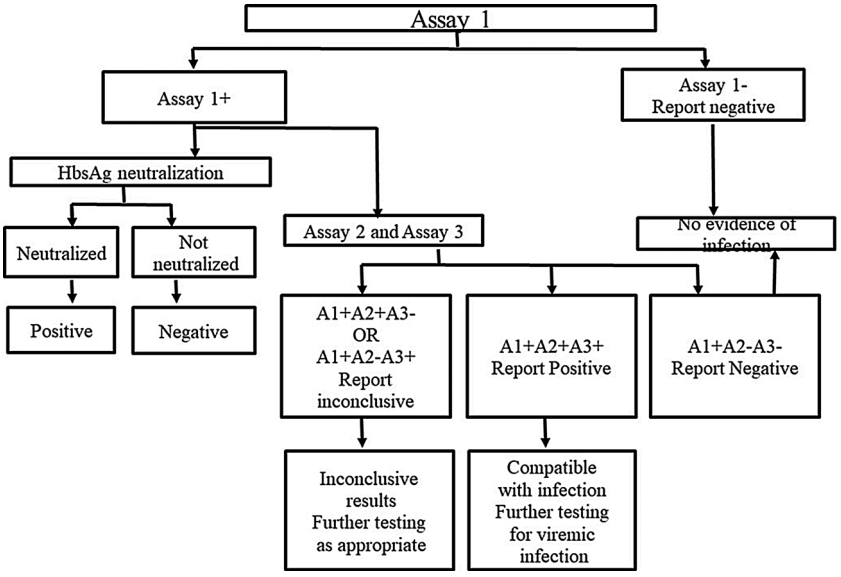

Fig. 3. Testing Strategy III decision tree. This testing strategy uses A1(ECLIA), A2(ELFA) and A3(RDT) to screen blood samples.

and VITROS immunodiagnostic products HBs Ag calibrator on the VITROS ECIQ and VITROS 3600 immunodiagnostic system. This method is based on the principle of specific antibody (anti-HBs) neutralization to confirm the presence of HBsAg. Two tests are re-run on A1 (one with neutralization reagents and one without neutralization reagents) and the difference in Signal to cut-off ratio) S/CO is compared to determine the neutralization of the antigen. A sample is confirmed positive if the result of non-neutralised sample is ≥0.80 S/CO and the neutralization is ≥50%.

Samples were initially tested for HBsAg by A1 (ECLIA). Samples testing non-reactive with A1 were reported as negative while those testing reactive were labelled as 'initial-reactive'. The initial reactive blood samples were aliquoted for further testing by A2 (ELFA) and A3 (RDT) assays. Confirmatory neutralization assay was performed on all initial reactive samples. Manufacturer's instructions were strictly followed while performing each assay. Donor notification and patient reporting were done on the basis of Strategy III (A1 + A2 + A3). The samples which were reactive by all three assays were interpreted as positive, those which were reactive by two assays (A1 + A2 or A1 + A3) were interpreted as inconclusive and the samples which were reactive only with the A1 were considered to be negative (false positive). The results of sequential serological assay testing strategy (I, IIA, IIB, and III) were compared with that of neutralization.

Data was entered and analysed in Microsoft excel (Ms-Office 2016, Microsoft, USA). Diagnostic evaluation was done for all four testing strategies. Positive Predictive value was calculated for each strategy with respect to gold standard.

This was an analysis of anonymized patient and donor testing records. Data did not have any personal identifiers and complete confidentiality was maintained.

A total of 112,011 blood samples (75,111 patient samples and 36,900 whole blood donor samples) were initially tested for HBsAg by A1 (ECLIA). From those, 1296 blood samples were found to be reactive by A1. As shown in Table 1, the number of test-results interpreted as positive reduced to 1188 with strategy IIA; 1078 with strategy IIB and 1074 with strategy III. The interpretation of the test results on the basis of strategy I, IIA, IIB and III was compared with the result of Gold Standard (Neutralization). We observed that the positive predictive value (PPV) of Strategy III > Strategy IIB > Strategy IIA > Strategy I.

Serological assays are commonly used as first line of testing strategy for screening of viral infections because of their relatively low cost, good turn-around-time as compared to nucleic acid tests. Among the commercially available HBsAg assays, ELISA and RDT are widely used for qualitative screening of HBsAg due to ease of use, shorter turn-around- time and lower cost (WHO, 2017). Chemiluminescence methods (ECLIA/CMIA) have been found to be efficient in detecting HBsAg with the advantages of high sensitivity and specificity compared to other tests (Peng et al., 2011). Therefore, we used ECLIA (A1) as the screening test. The screening test should have high sensitivity to ensure that no infected individual escapes detection; though this also means that there is some compromise with the specificity (and therefore diagnostic accuracy).

The reduced diagnostic accuracy of a single test may result in false positive (less often false negative) reporting. False positives can lead to unnecessary anxiety and increased cost associated with follow up tests and clinical assessment, while false negatives can result in further transmission of infection due to individuals not being referred for further assessment. Therefore, testing strategies have been devised to enhance the positive predictive value and diagnostic accuracy of the reported result.

Prevalence is the measure of total number of cases present during a specific time period for population at risk. It is the measure of disease burden for the specified population. If the prevalence of a disease is 1%, we would expect that on testing, one out of 100 people to be positive for the disease. Prevalence has an impact on positive predictive value (PPV) and negative predictive value (NPV) of tests. As prevalence increases, PPV also increases and NPV decreases if all other parameters are constant (Tenny and Hoffman, 2019). Parry et al. (2017) in their modelling exercise examined the diagnostic outcomes (PPV and NPV) as a function of prevalence for both one assay and two assays testing strategy. They demonstrated that single assay may result in considerable numbers of false positive results and low PPV in lower prevalence settings. Two assay testing strategy would result in PPV approaching 1 even at very low prevalence rates.

In our study where we applied strategy II (IIA & IIB) and III to HBsAg, we observed similar increments in the PPV of Strategy I < Strategy II < Strategy III as shown in Table 1. This PPV has been calculated in context of the confirmatory (neutralization test for HBsAg.

In our cost-benefit analysis, we observed that the costs per assay of the neutralization test was $26.18, and $2.18, $6.53, $3.05 and $7.40 for strategies I, IIA, IIB and III respectively. The cost of one neutralization test is reagents (diluent and antibody) plus the additional two A1 tests to complete the gold standard strategy. Neutralization tests also requires longer hands-on-time compared to other strategies. For example, adding RDT as A2 would involve the use of just a device and a shorter hands-on time.

Based on the comparison of strategies with the neutralization test, we can comprehend that a testing strategy which uses two or three testing assays sequentially shall detect more true positives obviating the need for a confirmatory test, which is more pertinent in a resource constrained setting.

In our analysis, the PPV of strategy III was the highest among the four testing strategies. The PPV rises significantly when we move from strategy I to strategy II; however, increments in PPV are marginal from strategy I to strategy II. Therefore, it would be prudent to adopt strategy II for diagnosis. Within strategy II, IIB (ECLIA + RDT) may be easier to perform than IIA (ECLIA + ELFA). While RDT does not require any equipment and can be performed by technical staff after minimal training, ELFA requires sophisticated equipment, specialized training and more elaborate controls and calibrators.

There are some inherent limitations to our analysis, as the subjects with inconclusive results were not followed-up; though ideally, they should have been followed-up and additional samples should have been submitted to tests. Although all the samples were tested with the first method, subsequent tests were performed only on initial reactive samples. Samples that tested negative on A1 assay were not subjected to A2/A3 assays; therefore, false negative rates could not be assessed. Blood donors constitute selected low-prevalence population and therefore results found in donor population cannot be applied to

Table 1
Comparison of PPV of four different strategies.

Assay Strategy	A1 (ECLIA)	A2 (ELFA)	A3 (RDT)	HBs Ag Positive	Gold Standard		Positive Predictive Value (PPV) align = "0in -3.8pt 10pt 7.1pt; text-align: center"
					Positive	Negative	
I	Reactive	NA	NA	1,296	1,173	123	90.5%
II A	Reactive	Reactive	NA	1,188	1,155	33	97.2%
II B	Reactive	NA	Reactive	1,078	1,050	28	97.4%
III	Reactive	Reactive	Reactive	1,074	1,048	26	97.6%

general population. This study suggests the use of reflex testing strategy comprising of either two-tests strategy or three-tests strategy. This may be further confirmed by employing larger studies.

In conclusion, sequential serological testing strategy comprising of initial sensitive test followed by more specific test increases the diagnostic accuracy of test report as compared to a single test.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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