

## **PRINCIPLES IN THE EVALUATION OF HBsAg TEST KITS: APPROPRIATE USE OF 2<sup>nd</sup> WHO INTERNATIONAL STANDARD (IS) AND REFERENCE PANEL FOR HBsAg**

One of the first viral markers to appear in blood following infection with HBV is hepatitis B surface antigen (HBsAg). This marker is usually detectable 2-8 weeks before biochemical evidence of liver dysfunction or the onset of jaundice and usually persists for several weeks. A proportion of individuals who are infected with HBV do not resolve the acute infection and become chronically infected. High levels of HBsAg are usually detected in their blood. Positive results in tests for HBsAg are therefore indicative of acute active-resolving infection or of a chronic-persistent state of infection.

Assay kits for diagnosis and for screening donor blood must be suitable for the purpose for which they are used. Testing for HBsAg has been performed with sensitive and specific immunoassays since 1973<sup>1</sup>. The needs of blood banks are somewhat different from the needs of diagnostic laboratories. In blood banks single testing of samples is conducted, and failures in detection will allow contaminated units to infect recipients. In the diagnostic setting, tests are usually undertaken in the context of clinical information where patients are suspected of being infected and where test results influence treatment and prophylactic strategies. Using the same test method, diagnostic laboratories are likely to find a far higher proportion of samples tested positive than blood banks. Kits used for epidemiological studies on prevalence or incidence of disease will have still different requirements.

### **Kit Performance, Accuracy**

The main characteristics that define performance of diagnostic assay kits are *sensitivity* and *specificity*. *Diagnostic sensitivity* of HBsAg test kits is defined as the ability of a test to detect as many HBV-infected patients as possible, including patients with low serum levels of the antigen. Manufacturers should evaluate sensitivity of a new kit by testing 2-3 00 samples known to be positive with well-established kits. Some specimens are weak positives because they are from patients in early stages of infection, the seroconversion or “window period.” During this period, an individual may not have manifested any clear symptoms. Because weakly positive HBsAg specimens are typically uncommon, scientists attempt to simulate them by making dilutions of a known HBsAg-positive serum. Laboratories can assess sensitivity in a quantitative way by measuring a kit’s ability to detect highest dilutions. However, it must be recognized that the correlation between low titer analytical specimens and seroconversion samples is loose. The detection of antigen at limiting dilutions is most accurately referred to as the kit’s *analytical sensitivity*.

Specificity is the ability of a kit to score a sample accurately. Specificity refers to negatives being accurately detected as non-reactive. Highly specific kits have good reproducibility and will give a low proportion of non-repeatable positives. Manufacturers will assess specificity of a new test kit by testing specimens from 3-5 thousand blood donors. Since the vast majority of donors are expected to be negative for HBV, most of them should be found non-reactive with the test kit when the test is highly specific.

### **Licensing Practices**

Policies regarding evaluation of test kits vary widely from country to country. Some countries require that commercial kits undergo a licensing procedure before they can be used. Manufacturers are required to submit a license application that includes data on sensitivity and specificity as outlined above as well as detailed information on other items such as data on reproducibility and stability. Some countries also require that each production lot be subjected to a batch release procedure before a batch (“lot”) is released.

Unfortunately, many countries have no licensing procedure to evaluate or control the kits that are offered for sale, and they may have no infrastructure to do this. When this is the case, the user of kits must rely on the claims of the manufacturer regarding their performance, and quality is dependent on the quality assurance programs implemented by the manufacturer. Information on sensitivity and specificity supplied in package inserts could be misleading if studies under a variety of field conditions have not been not performed and carefully evaluated.

## REFERENCE MATERIALS

Reference materials for evaluating diagnostic kits that detect viral markers in blood and plasma units are used primarily for assessing sensitivity and specificity. A typical collection of reference materials includes:

- 1.) Seroconversion panels: These are a series of serum samples taken from the same individual at various times following a patient's exposure to HBV. Several of these panels directly facilitate the evaluation of diagnostic sensitivity in assays for the detection of antigens (or antibodies). However, since it is seldom possible to know when a patient has been exposed, the acquisition of seroconversion panels is very difficult; they are usually in short supply worldwide and are very expensive.
- 2.) Control sera and working standards: Many of the *in-vitro* diagnostic (IVD) assays are quantitative and base their measurements on mathematical calculations relative to calibrators. Control sera or working standards that are included in every assay (or need to be ordered separately) are used to verify calibration. The working standards may be used as run controls to monitor the reproducibility of assays on a daily basis. Tests for HBsAg are usually not intended for quantitative applications. In these kits, control sera may be used in the calculation of cut-off and thus can ensure that an adequate level of sensitivity is being achieved in every test. Calibrators and controls are developed by the manufacturers themselves, but the unitage assigned to them should be based on international reference standards.
3. Other specimens: Evaluation of new kits should include panels of difficult (clinical) samples and panels of local HBsAg samples representing the prevalent HBV genotypes and variants in that target region. Those samples are often not available to the regulatory authorities (RA) and are usually obtained and tested by the manufacturers. Data obtained on these collected panels are included in the dossier submitted to the RA.

### International Reference Materials

Traditionally, International Standards have been established to facilitate comparison of results from different laboratories during standardization of the biological activity of therapeutic products or reagents. However, the use of well-characterized control materials produced and supplied by an independent organization will also give laboratories who use kits for detecting markers of infection an assurance of performance via qualitative and quantitative data.

WHO provides International Reference Materials that contribute to the global harmonization of regulations applying to assays used for blood screening and patient diagnosis. These quantified reference standards assure reliable and constant results of *in-vitro* diagnostic devices (IVDs) and allow for the communication of results in a common language worldwide.

With the collaboration of the WHO Regional Offices, regional networks of regulatory authorities and reference laboratories will be supported by provision of the WHO Reference

Materials. Both WHO Preparations, the 2<sup>nd</sup> HBsAg International Standard (IS) and the appendant HBsAg Reference Panel, are intended as quantitative reference standards for HBsAg.

## **2<sup>nd</sup> International HBsAg Standard**

The WHO 2<sup>nd</sup> International Standard for HBsAg was established in 2003 and is intended for the calibration of secondary reference materials of HBsAg by manufacturers and national regulatory authorities. The donation chosen for the preparation of the panel was an antigen-positive serum, HBsAg genotype A, subtype adw2. It was also anti-HBe positive, but was negative for HCV RNA, anti-HCV, and anti-HIV 1+2.

A WHO collaborative study of six participants calibrated the 2<sup>nd</sup> IS vs. 1<sup>st</sup> IS using eleven different HBsAg immunoassays with the intention of preserving as closely as possible the value of the unit from the 1<sup>st</sup> IS<sup>2,3</sup>. The coefficients of variation observed among these kits was 30.0% with a range in values from 22.0 to 47.3 IU<sup>4,5</sup>. A mean value of 33 IU/vial was assigned to the 2<sup>nd</sup> HBsAg IS.

The collaborative study showed that the IS is effective in assessing the analytical sensitivities of HBsAg kits worldwide; titration of the 2<sup>nd</sup> IS with any of the types of kits used worldwide will give an accurate and objective indication of its analytical sensitivity.

## **Reference Panel**

The WHO Reference Panel is intended to aid national regulatory authorities in evaluating the analytical sensitivity of rapid tests for HBsAg. The Panel derives from the 2<sup>nd</sup> International Standard and is composed of four panel members (A - D) comprising different concentrations of HBsAg as well as a negative sample (E). Panel members A, B, C and D were prepared in negative serum at concentrations of 8.25, 2.06, 0.52 and 0.13 IU/vial respectively. The Panel is of particular importance in areas where the required human serum diluent (i.e., negative for HBsAg, anti-HBs, anti-HCV and anti-HIV) is scarce.

In a second phase of the WHO Collaborative Study, 10 international laboratories tested the IS candidate and panel using 20 other immunoassays and rapid tests. The study showed that the range of concentrations of HBsAg in the prediluted panel was suitable for evaluating a wide range of assay kits from around the world. It provided data that distinguished between test kits with high sensitivity from those with low sensitivity. The data show that the Panel provides a convenient resource for authorities to assess analytical sensitivity, especially of rapid tests.

The use of the Reference Panel will permit laboratories, organizations and regional regulatory authorities to set requirements for the analytical sensitivity of different assays and batches of the same kit. Using these reagents to monitor the performance of different batches of an individual kit over a period of time will provide information on batch-to-batch variation, so that range limits can be established.

Initially it is envisaged that the WHO Reference Panel will be distributed to reference laboratories so that they can periodically assay representative lots of kits available for use in each region. Eventually it is expected that such activities will be conducted independently on a national level.

## **USE OF INTERNATIONAL UNITS (IU) INSTEAD OF "NANOGRAM (ng)"**

Many workers in the hepatitis B field still use the nanogram (“ng”) when quantifying sensitivity of HBsAg kits. Continuing this practice is undesirable because different organizations have based their ng calculations on HBsAg with widely different biochemical and immunologic activity<sup>6,7</sup>. Indeed, considerable differences between ng assignments of several secondary standards were revealed during the WHO collaborative study that established the 2<sup>nd</sup> HBsAg International Standard<sup>3</sup>.

Due to those differences it is not possible to give a reliable factor for the conversion of ng to IU. Rather, replacement studies are required for every test kit whose analytical sensitivity has been measured in ng. These studies are to be performed as parallel-line assays<sup>8</sup> similar to those performed for establishing any secondary standard or new dilution series.

## LIMITATION OF USE

The quantitative detection of different subtypes of HBsAg may vary because different commercial kits may be detecting different epitopes. It is therefore possible that the use of different assay kits may result in the assignment of different values to the panel members even when the International Standard is assayed concurrently and appropriate statistical analysis by the parallel-line method applied<sup>8</sup>. The influence of the genotypes in the evaluation of the quality of the HBsAg diagnostic devices will be examined by a WHO study planned for the near future.

## REFERENCES

1. Overby LR, Miller JP, Smith ID, Decker RH, Ling C-M. Radioimmunoassay of hepatitis B virus-associated (Australia antigen) employing <sup>125</sup>I-antibody. *Vox Sanguinis* 1973; 24: 102-113.
2. Seagroatt V, Ferguson M, Magrath DI, Schild GC, Cameron CH. The British reference preparation of hepatitis B surface antigen. *Lancet* 1982 (ii) 391-392.
3. Hepatitis B surface antigen (subtype ad). WHO Technical Report Series 1987; 745: 18.
4. WHO Working Group on International Reference Preparations for Testing Diagnostic Kits Used in the Detection of Hepatitis and HIV Diagnostic Kits: Report of a collaborative study to 1) assess the suitability of a candidate replacement International Standard for HBsAg and a reference panel for HBsAg and 2) to calibrate the candidate standard in IU (WHO/BS/03.1987); adopted by ECBS 2003.
5. WHO Working Group on International Reference Preparations for Testing Diagnostic Kits used in the Detection of HBsAg and anti-HCV Antibodies (October 2003).
6. Barbara JAJ. Questions of quality: How much HBsAg is there in this sample and is our assay sensitive enough to detect it. *Vox Sanguinis* 1993; 65, 249-250.
7. Gerlich W, Thomssen R. Standardised detection of hepatitis B surface antigen: determination of its serum concentration in weight units per volume. *Developments in biological standardization* 1975; 30:78-87
8. Finney DJ. *Statistical methods in biological assay*. 3rd ed., London: Charles Griffin 1978.