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Development of in-house Enzyme-Linked-Immunosorbent-Assay (ELISA) for determination of antibody to hepatitis B surface antigen

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Vaccination programmes are considered as the most important public health measure in the prevention and control of hepatitis B infection. Testing for the antibody status of the individual to hepatitis B is needed to further determine the response of hepatitis B vaccine. An in-house two-stage Enzyme Linked Immunosorbent Assay (ELISA) system was developed using locally purified hepatitis B surface antigen (HBsAg) as the solid phase and commercially available anti-human IgG horse radish peroxidase as the conjugate. Checkerboard titration was carried out and the dilutions determined. Using MONOLISA ELISA system as the standard, the sensitivity and the specificity of the in-house ELISA system was found to be 73% and 98%, respectively. The positive and negative predictive values were 85% and 96%, respectively.

INTRODUCTION

Hepatitis B virus (HBV) infection is an important health problem worldwide, as the infection can cause significant liver diseases that range from fulminating hepatitis to hepatocellular carcinoma. HBV infection in the Asia-Pacific region is among the highest in the world, and chronic HBV infection in most of the countries of the Asia-Pacific region including Myanmar is high (>10% prevalence) [1]. WHO has estimated that 12% of the Myanmar population could carry HBV and 28/100,000 population could develop hepatocellular carcinoma [2]. Accordingly, HBV infection has been considered as a priority health problem in Myanmar and health authorities have taken various steps to control and manage HBV infection. It has been shown that vertical transmission at the perinatal period plays a major role in the acquisition of HBV infection in Myanmar. Twenty five percent of children born to hepatitis B surface antigen positive mothers and up to 60% of children born to HBsAg and HBeAg positive mothers became HBsAg positive within the first year of life [3]. Thus, immunization of newborns with hepatitis B vaccine is an important strategy for control of hepatitis B in Myanmar. Incorporation of hepatitis B vaccine into the Expanded Programme of Immunization (EPI) programme has been initiated and is achieving good results.

Serology is the main diagnostic and prognostic tool for HBV infection. The presence of antibody to hepatitis B surface antigen (anti-HBs) signifies the person's previous exposure to hepatitis B virus or hepatitis B vaccine and denotes immunity to the hepatitis B virus and protection against re-infection. The absence of anti-HBs reveals the person's susceptibility to HBV infection. With the introduction of HBV vaccine in Myanmar, the need of testing for the antibody status of the individual to hepatitis B is increasing as the detection of

anti-HBs will be of great help in determination of the response to HBV vaccine. It will also assist the health care providers to assess the successes and failures of the immunization programmes. However, commercial test kits for determination of anti-HBs are relatively expensive in Myanmar. Thus there is a need to develop a locally available anti-HBs ELISA system to reduce the cost of the antibody testing.

The objective of the present study is to develop a test system to determine the antibody to hepatitis B surface antigen and to evaluate the test system against the commercially available ELISA.

MATERIAL AND METHODS

Test principle of the developed HBsAg ELISA kit

Detection of the hepatitis B surface antibody by enzyme linked immunosorbent assay (ELISA) was carried out using the solid phase immunoassay principle. Hepatitis B surface antibody present in the serum sample was bound to purified HBsAg immobilized on the surface of the polystyrene wells. After washing, the conjugate (anti-human IgG-horseradish peroxidase) was added and this conjugate was attached to the hepatitis B surface antibody in the serum sample which is captured by the purified HBsAg immobilized on the polystyrene wells. The excess conjugate was removed by washing and the enzyme activity was determined by action on the substrate, orthophenylene diamine dihydrochloride (OPD). The reaction was stopped by adding 4 N surphuric acid. The intensity of the yellow-orange color developed by the reaction was proportional to the concentration of hepatitis B surface antibody in the sample.

Purification of HBsAg

Purification of HBsAg from plasma of apparently healthy chronic HBsAg carriers by the New York Blood Centre method includes the following steps; collection of high titre HBsAg positive, HIV and Hepatitis C negative plasma, acidification, precipitation, absorption, concentration, isopyknic centrifugation, and dialysis. The purified HBsAg sample was further analysed by SDS-PAGE for purity.

Checker board titration

Checker board titration was done by using different dilutions of purified HBsAg from the human blood as coating antigen and different dilutions of anti-human IgG HRP conjugate (Sigma Co.) as the conjugate in the ELISA system. Positive and negative samples were used in duplicate for each dilution of coating antigen and conjugated antibody.

Evaluation of the in-house anti-HBs ELISA test

The in-house anti-HBs ELISA system was evaluated against a commercially available ELISA test kit , (MONOLISA ELISA, USA). The samples were coded, randomly rearranged and blinded to the person who carried out the ELISA tests.

Samples

Serum samples were collected from persons attending the Vaccine and Diagnostic Clinic of the Department of Medical Research (Lower Myanmar). One hundred serum samples, each of 2 ml in volume, from subjects consecutively recruited were included. They were tested for the presence of HBsAg by in-house hepatitis B surface antigen ELISA [4]. Nine samples were positive and the remaining 91 samples were further tested for anti-HBs seropositivity by the MONOLISA ELISA and in-house anti-HBs ELISA tests.

Statistical Methods

Data analysis was performed with SPSS (Statistical Package for Social Scientists Ver 10.1; SPSS Corporation, Chicago, IL, U.S.A.) on a IBM computer. Sensitivity and specificity and their confidence intervals were calculated [5].

RESULTS

The results of the checker board titration are shown in Table 1. The optimal dilution (d value) was obtained by calculating the greatest difference between the O.D. of the positive and negative samples. The " d " value had to be > 0.21 to be valid. The optimal dilution of the coating and conjugate were found to be 1/80 and 1/5,000 respectively.

Table 1. Results of the checker board titration

Anti - human IgG HRP conjugate		Coating purified HBsAg							
		1/20		1/40		1/80		1/160	
		1	2	3	4	5	6	7	8
1/ 5,000	A	0.39	1.04	0.35	1.18	**0.39**	**1.25**	0.39	1.27
	B	0.27	0.91	0.27	0.93	**0.31**	**1.09**	0.34	1.10
1/ 10,000	C	0.23	0.64	0.20	0.65	0.22	0.62	0.23	0.72
	D	0.21	0.59	0.22	0.65	0.26	0.68	0.25	0.71
1/ 20,000	E	0.13	0.31	0.13	0.32	0.12	0.33	0.13	0.38
	F	0.10	0.31	0.11	0.33	0.12	0.28	0.13	0.37
1/ 40,000	G	0.07	0.14	0.07	0.18	0.08	0.17	0.08	0.21
	H	0.07	0.10	0.07	0.19	0.08	0.20	0.09	0.21
		-	+	-	+	-	+	-	+

A total of 91 samples were tested by both commercial anti-HBs ELISA kit (MONOLISA) and the developed in-house anti-HBs ELISA kit. The results are shown in Table 2.

Table 2. Comparison of results between the two tests

		MONOLISA ELISA		
		+	-	TOTAL
In-house ELISA	+	8	2	10
	-	3	78	81
	TOTAL	11	80	91

Seventy eight samples were negative and 8 samples were positive by both tests. There were three samples that were positive by the commercial kit and negative with the in-house ELISA kit. If we considered the commercial kit as a gold standard against which we compare our test, the results were as follows: sensitivity 73% (95% CI 60% to 86%), specificity 98% (95% CI 95% to 99%), positive predictive value 85%, and negative predictive value 96%.

DISCUSSION

Hepatitis B virus (HBV) infection is an important health problem worldwide and vaccination programmes are considered as the most important public health measure in the prevention and control of hepatitis B infection. DMR has successfully produced the plasma-derived hepatitis B vaccine to be used in prevention of HBV infection [6]. Preventing HBV transmission during early childhood is important because of the high likelihood of chronic HBV infection and chronic liver disease that occurs when children less than 5 years of age become infected [7]. Integrating hepatitis B vaccine into childhood vaccination schedules in populations with high rates of childhood infection (e.g., Alaskan Natives and Pacific Islanders) has been shown to interrupt HBV transmission [8]. With introduction of vaccination programmes it is necessary to monitor the anti-HBs seropositivity, as in vaccination the desired immune response to vaccines is the production of antibodies.

Antibody to hepatitis B surface antigen is a marker of immunity. Its presence indicates an immune response to HBV infection, an immune response to vaccination, or the presence of passively acquired antibody. The most widely used anti-HBs determination test kits worldwide are ELISA as they are most appropiate for screening large numbers of samples on a daily basis. However, the ELISA system needs equipment such as ELISA Readers to perform the assays. The developed in-house ELISA might be practical only in some laboratories as trained laboratory personnel and some equipment to perform the procedure are needed. Commercially available immuno-chromatographic tests are much simple to perform and advanced training programme is not needed to perform the tests. However, the ICT tests usually

indicate qualitative presence of the antibody to hepatitis B surface antigen and quantitative levels can be determined mostly by use of ELISA test systems together with standard anti-HBs. Previously, researchers from the Department of Medical Research (Lower Myanmar) had developed an in-house radioimmunoassay system to detect anti-HBs. The procedure involved the use of polyvinylchloride microtitre plates as solid phase and purified human hepatitis B surface antigen was used for coating microtitre plates and for radioiodination [9]. Although the assay is semiquantitative, the difficulty in obtaining iodine-125 radio-isotope has limited its use in Myanmar.

An adequate (protective) antibody response has been defined as greater than or equal to 10 sample ratio units (SRU {sample signal divided by the test kit negative control mean}) by radioimmunoassay (RIA) [10]. Subsequently, the determination of anti-HBs levels was standardized by expressing anti-HBs concentrations in milli-international units per milliliter (mIU/mL) using the WHO Anti-HBs Reference Preparation [11]. Because the value of 10 SRU by RIA and the manufacturers' recommended positive threshold for enzyme immunoassays (EIA) were similar to 10 mIU/mL, Immunization Practices Advisory Committee (ACIP) recommendations issued in 1987 defined the protective level of anti-HBs as greater than or equal to 10 mIU/mL, approximately equivalent to 10 SRU by RIA or positive by EIA [12]. The positive results which were inparallel with positivity of MONO-LISA ELISA were regarded as equal to or more than 10 mIU/mL. It was found that of 11 samples positive by MONOLISA ELISA, 8 were positive by the in-house ELISA (74%).

Radioimmunoassay test system is the gold standard against which the test kit should be evaluated. However, since it was not available, for this present study a commercially available ELISA test system was used to evaluate the developed in-house test. It was found that the ELISA could be used for determination of circulating antibodies to hepatitis B surface antigen. However, the sensitivity of the in-house system is much lower (73%) than the commercially available test kits. The poor sensitivity limits the use of this in-house ELISA in sero-epidemiological studies.

The developed in-house anti-HBs ELISA is economical as it is based on locally produced HbsAg as the coating antigen. It. has high specificity but the sensitivity needs to be improved. It could be useful in testing the immune response in persons who have received hepatitis B vaccine but further optimization is needed for use in screening of persons in seroepidemiological studies.

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