DEVELOPMENT OF AN ELISA KIT FOR DETECTION OF HEPATITIS B SURFACE ANTIGEN IN PLASMA AND SERUM, BASED ON POLYCLONAL ANTIBODIES GENERATED IN KEMRI, KENYA

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DEVELOPMENT OF AN ELISA KIT FOR DETECTION OF HEPATITIS B SURFACE ANTIGEN IN PLASMA AND SERUM, BASED ON POLYCLONAL ANTIBODIES GENERATED IN KEMRI, KENYA

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2009

DECLARATION

This thesis is my original work and has not been presented for a degree in any other University

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This thesis has been submitted for examination with our approval as University supervisors

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DEDICATION

I dedicate this thesis to my wife, Margaret, my children Joy, Jesse and Mary-Christine and the staff of KEMRI Production Department, specifically Mr. Samuel Muchiri for his kind inspiration and exceptional support.

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ABBREVIATIONS AND ACRONYMS

Ab	Antibody
ABTS	2, 2-azino-di (3-ethyl-benzathiazoline) sulphonic acid
Ag	Antigen
Anti-HBs	Hepatitis B surface Antibody
AP	Alkaline phosphatase
BSA	Bovine Serum Albumin
BSAT	Bovine Serum Albumin + Tween 20
DAC ELISA	Dual Antibody Capture ELISA
DAS ELISA	Dual Antibody Sandwich ELISA
DNA	Deoxyribonucleic acid
D-SN	Diagnostic sensitivity
D-SP	Diagnostic specificity
EIA	Enzyme Immuno Assay
ELISA	Enzyme Linked Immunosorbent Assay
Fab	Antibody binding fragment
Fc	Fragment that is easily crystallized
HBcAg	Hepatitis B core Antigen
HBeAg	Hepatitis B e Antigen
HBsAg	Hepatitis B surface Antigen
HBV	Hepatitis B Virus
HRP	Horse Radish Peroxidase
KEMRI	Kenya Medical Research Institute
ng	Nanogram
NGPS	Normal Guinea Pig Serum
Nm	Nanometer
NPHLS	National Public Health Laboratory services
NPV	Negative Predictive value
NRS	Normal Rabbit Serum
OD	Optical Density
PBS	Phosphate Buffered Saline

PCR	Polymerase Chain Reaction
pH	Hydrogen Potential
PPV	Positive Predictive value
R-PHA	Reverse Passive Haemagglutination
SOP	Standard Operating Procedure
TMB	3, 3', 5, 5'tetramethylbenzidine
UV	Ultraviolet
WHO	World Health Organization

ABSTRACT

Hepatitis B infection is a disease of the liver caused by Hepatitis B Virus (HBV), a double-stranded DNA virus coated with an envelope containing Hepatitis B surface antigens (HBsAg). HBsAg levels in blood are high as long as the viral particles continue to exist in the liver cells. Hence they are the most important markers used in screening for the presence of Hepatitis B infection in many of the diagnostic test kits in the market. The currently available ELISA diagnostic kits for HBV are both imported and expensive.

The main objective of this study was to develop a cost-effective Enzyme-linked Immunosorbent Assay (ELISA) kit for detection of HBsAg in plasma and serum using polyclonal antibodies produced in KEMRI.

The capture polyclonal antibodies were obtained from KEMRI Production department where they had been raised in guinea pigs inoculated with locally prepared HBsAg. The Sandwich-based ELISA system was prepared by coating capture antibodies on 96-well microplates and blocking the void spaces using the Bovine Serum Albumin and Tween 20 (BSAT) blocking buffer after which samples were applied. The Horse Radish Peroxide (HRP)-linked ovine HBsAg detection antibodies were incubated in the wells and washed with Tween 20 Wash Buffer to remove the unbound antibodies. The 3, 3', 5, 5' tetramethylbenzidine (TMB)-hydrogen peroxide substrate was incubated for a 30 minutes and absorbance values read using an ELISA plate reader. The reagents used in preparation of this kit were optimized using the ELISA checkerboard technique.

The developed kit was assessed and found to have diagnostic sensitivity of 96.1%, diagnostic specificity of 100%, positive predictive value of 100% and negative predictive value of 95.7% with Hepanostika Ultra HBsAg kit as a gold standard. The analytical

sensitivity of the kit was found to be 4.62ng/ml and no analytical non-specificities were noted in samples positive for HIV and HCV respectively. The kit showed desirable repeatability profile with the coefficient of variance of intra-run repeatability of 1.38% and inter-run repeatability of 5.3% against the limit of 20%. The overall inter-observer variation agreement, Kappa statistic, was found to be 1 (one) signifying perfect correlation while the Pearson correlation coefficient (r^2) to determine correlation between values measured by different assays were 0.917, 0.939 and 1.00 against three established kits.

The ELISA kit developed in this study will be tested further in the field for a period of one year before it is applied for registration at the National Public Health Laboratory Services, Nairobi. It will also be validated against a number of mutagenic cysteine variants and subtypes on the "a" determinants of HBsAg.

CHAPTER ONE

1.0 INTRODUCTION

1.1. Background information

Three hundred and fifty million people are chronically infected with Hepatitis B Virus (HB) worldwide with 600,000 dying each year due to its acute or chronic consequences (WHO, 2007). Kenya is a high hepatitis B virus endemic zone, with an average carrier rate of 7-10% and the range of 3- 30% (Okoth *et al.*, 1990). Crowther (2001a) describes ELISA analytical system as a technique in which an enzyme is used as a label on an antigen or antibody that binds to the antigen (or antibody) of interest (analyte). After binding, the enzyme portion is assayed to allow for detection of an immune reaction and the estimation of analyte. According to Crowther (2001a) and Keith *et al.* (2006), ELISA systems can either be in homogeneous or heterogeneous formats with former not requiring separation of bound and free analyte in order to detect or measure the target antigen while the latter requires their separation (Kurdziel *et al.*, 2001). The two formats can belong to the three major classes of ELISA namely direct, indirect, and sandwich classes (Crowther, 2001a). The three ELISA classes can in turn be divided into Competition or Inhibition ELISA (Crowther, 2001b).

Biotin-Avidin ELISA, ELISA Reverse method and device and Multiplex ELISA are three other classes of ELISAs that utilize previously described format. Biotin-Avidin ELISA system, described by Jordan (2005) uses directly-labelled, enzyme-tagged primary and secondary antibodies for detection of analytes and can achieve substantial amplification over the other methods due to the fact that many biotin molecules can be coupled to the

antibody, enabling the biotinylated antibody to bind more than one molecule of avidin coupled to the enzyme. ELISA Reverse method and device described by Eberle *et al*. (1992) uses a solid phase made up of an immunosorbent polystyrene rod with 4-12 protruding ogives (curved shape) where the entire device is immersed in a test tube containing the collected sample. The process is followed by steps of washing, incubation in conjugate and incubation then chromogen. Multiplex ELISA (Virella and Litwin, 2007; Gonzalez *et al.,* 2008) is a relatively newer technique that involves a protein array format that allows simultaneous detection of multiple analytes at multiple array addresses within a single well.

ELISA test kit can be developed using either polyclonal antibodies or monoclonal antibodies (Atassi *et al.*, 1984). Monoclonal antibodies are more specific, reproducible and purer but are generally more expensive to produce (Okoth *et al.,* 1999). Polyclonal antibodies have the advantage of being able to detect variant forms of the antigens (Jongerius *et al*., 1997; Louisirirotchanakul and Kanoksinsombat, 2006).

Cross-activity of antibodies, which occurs when an epitope is shared between the desired antigen and an irrelevant antigen, can either be due to similar epitopes, in which case it can be reduced by allowing longer incubation time or by purifying the antigen (KPL, 2008). If the cross-activity is due to the presence of anti-Ig antibodies it can be reduced by nonspecific antibody from the species used to generate the antibody (Thomson and Ketterhagen, 1984; KPL, 2008). Monoclonal antibodies are also known to show some cross-reactivity as they can cross reacts with epitopes having a similar three dimensional structure (KPL, 2008).

Most of the ELISA plates adsorb proteins passively (Crowther, 2001a) but in special cases hydrophobic bonds cannot be formed and covalent linkages are used (Crowther, 2001a). Small peptides can be adsorbed on the plate but their epitopes are occasionally too few to be detected unless they are attached to large proteins through a spacer arm Palfreyman *et al.* (1984).

The blocking reagents block hydrophobic sites that are left unoccupied when an immunoreactant is applied on the ELISA plate. They prevent non-specific binding of subsequent reactants that could result in high background signal, low specificity and sensitivity (Zola, 1987; Crowther, 2001a). The blocking agents are mostly either detergent such Tween 20 and Triton X-100 or proteins such as Bovine Serum Albumin (BSA), Non-fat dry milk (NFDM), Normal serum, Casein or Caseinate and Fish gelatin. The washing buffers commonly used in ELISA include PBS, Tris saline and imidazole-buffered saline and they are sometimes used together with detergents such as Tween 20 and Triton X-100 and with a protein such as BSA when there are problems with high background signals (Crowther, 2001b). The most widely used detection molecules in ELISA are the enzymes Horse Radish Peroxidase and Alkaline Phosphatase and they mostly used with the two chromogen substrate - 3, 3', 5, 5' tetramethylbenzidine (TMB) and 2,2'-azino-di-(3 ethylbenzthiazoline sulfonic acid) (ABTS) (Goars, 1983).

The optimal concentrations of various reagents such as capture, detection and conjugated antibodies in the development of ELISA kits are optimized by using Checkerboard titration assays where two or more components are varied in a way that results in a pattern (Chart *et al.,* 1994; Pascho *et al*., 1997 and RD System, 2008).

Cut-off points of the ELISA are determined based on the following methods among others: the frequency distributions of test results from uninfected and infected reference animals (Zweig and Campbell, 1993; Jacobson, 1996 and Fawcet, 2006), uninfected reference animals (Greiner *et al.*, 1994) and test results from sera drawn randomly from within the target population with no prior knowledge of the animals' infection status (Greiner *et al.*, 1994). Repeatability tests are carried when the initial stage of development of the ELISA is nearly complete Gao *et al.* (2004) and Jacobson (1996). The performance of the developed kit is assessed by determining the analytical sensitivity, analytical specificity, diagnostic sensitivity and diagnostic specificity in ELISA (Jacobson, 1996; Crowther, 2001a; Van Roosmalen *et al.*, 2006).

There are a number of diagnostic kits for detection of HBsAg in body fluids in use worldwide, which are based on various technologies such as particles agglutination, hemogglutination, immunochromatography, ELISA and PCR (Howard *et al.*, 2005a). The most extensively used HBsAg screening tests are ELISAs as they are suitable for screening large numbers of specimens on daily basis, as is the case in blood transfusion services (WHO/BCT/BTS/01.4, 2001). However, a number of blood transfusion services in resource limited countries do not use ELISA kits always due to relatively higher costs and instead cheaper kits such as rapid Immunochromatographic kits are used (WHO, 2007).

In late 1990's Kenya Medical Research Institute (KEMRI) in collaboration with Japan International Cooperation Agency (JICA) developed an affordable and relatively effective diagnostic kit for detection of HBsAg in plasma/serum based on the Reverse

Particle Haemogglutination (RPHA) technology (Okoth *et al.*, 1999). The initiative to develop this kit arose from the genuine concern that, although the prevalence rate of the HBV in Kenya was high (7-10%), donors' blood for transfusion was not routinely screened for HBV at that time due to high costs of the HBV screening tests (Okoth *et al.*, 1999).

After the 1998 bombing of the USA Embassy in Nairobi by terrorists, the United States Agency for International Development (USAID) and Family Health International (FHI) initiated massive support to the National Blood Transfusion Centers in Kenya. This support is still sustained and it includes, among others, supply of the blood screening kits such as ELISA kits for HBV (Abdalla *et al.*, 2007). Unfortunately, over-reliance on donors to provide full support in such a critical area of the national health system requires caution as donor fatigue can set in without notice as recently reported in a press release by the Kenya Red Cross that "Donor fatigue has been on the rise; hence the need to develop alternative funding opportunities and avenues to strengthen the disaster preparedness and response" (Kenya Red Cross, 2008).

The development of a HBV ELISA kit with the performance profile comparable to similar imported kits would provide fall back measures to cushion the nation from such eventualities as well providing affordable alternative to those who cannot currently afford expensive HBV ELISA test kits.

1.2 Research Problem

The ELISA testing kits that are currently used in Kenya for screening blood for Hepatitis B Virus are imported and expensive. Most of them are donated and the sustainability of their supply is not fully assured. Hence a need of developing a cheap and effective ELISA test kits for screening blood for HBV using the locally produced polyclonal antibodies.

1.3 Justification

In Kenya, HBV infection is endemic with the average carrier rate of 7-10% (Okoth *et al.*, 1999) and prevalence of 2.5% among blood donors (Makokha, *et al.,* 2004). To avoid transmissions of this virus through blood transfusion, all donated blood must be screened for the presence of HBsAg. ELISA kits that detect HBsAg are widely used for this purpose as they are generally sensitive, specific and robust (Crowther, 2001a). In Kenya, these kits are imported, expensive and mainly acquired through the assistance of foreign donors (Abdalla *et al.,* 2007). Over reliance on donors to provide such critical products for the country's health system is an undesirable situation as donor fatigue and other unfavourable conditions can arise without adequate warning (Kenya Red Cross, 2008). KEMRI has developed the technology and capacity to produce anti-HBsAg polyclonal antibodies. These antibodies have been used to manufacture and commercialize a cheap and effective Reverse Passive Haemagglutination (RPHA) HBsAg detection kit, HEPCELL® (Okoth *et al.*, 1999).

This project was designed to use these polyclonal anti-HBs generated by KEMRI to produce an ELISA kit for detection of HBsAg in plasma / serum that is cost-effective,

readily available with quality attributes comparable to similar ELISA kits in use in Kenya. The developed ELISA kit will be cheaper than RPHA Hepcell in additional to having the desirable properties of ELISA testing system of being more sensitive, specific and robust

1.4 Hypotheses

1.4.1 Null hypothesis

It is not possible to develop an ELISA kit for detection of HBsAg in plasma and serum using locally produced antibodies

1.4.2 Alternative Hypotheses

It is possible to develop an ELISA kit for detection of HBsAg in plasma and serum using locally produced antibodies

1.5 Objectives

1.5.1 General Objective

To develop an effective ELISA kit for detection of HBsAg in plasma and serum using polyclonal antibodies generated in KEMRI

1.5.2 Specific Objectives

i. To develop an ELISA Kit for detection of HBsAg in plasma and serum using locally produced polyclonal antibodies

ii. To compare the diagnostic sensitivity of the commonly used HBV testing kits in Kenya

iii. To compare the cost of the developed kit with similar kits in the local market

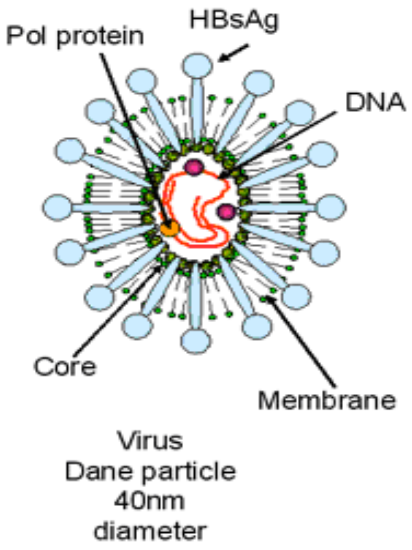
CHAPTER TWO

2 LITERATURE REVIEW

2.1 Hepatitis B Infection and Virus

Hepatitis B Virus (HBV) infection is a disease of global epidemiological concern, with 2 billions people having been infected with the virus. (WHO, 2007). Close to 25% of adults who become chronically infected during childhood later die from liver cancer or cirrhosis caused by the chronic infection (WHO, 2007). The endemicity of Hepatitis infection varies geographically and it is said to be high when chronic infection prevalence is 8%-15% (Cluster *et al.*, 2004). In Kenya, in areas of low endemicity, 40-60% of adults have HBV markers and the average carrier rate is 7-10% with the range of 3-30% (Okoth *et al.*, 1990).

HBV is a double-stranded DNA virus of an average diameter of 40nm, belonging to the family hepadnaviridae and coated with an envelope which contains the lipoproteins from the host cell and HBsAg embedded on the surface (Landers *et al.,* 1977; WHO/BCT/BTS/01.4, 2001; Howard *et al.,* 2005b), Figure 2.1. The genome of the virus is closely associated with the polymerase protein (P) which is surrounded by the core antigens, HBcAg and HBeAg. During replication substantial HBsAg is shed into the body fluids, especially the blood system, in form of spherical particles of diameter of about 20nm and filamentous particles of up to 200nm long (Dane *et al.,* 1970, University of South Carolina, School of medicine, 2007).



Adapted from "University of South Carolina, School of medicine notes" (2007)

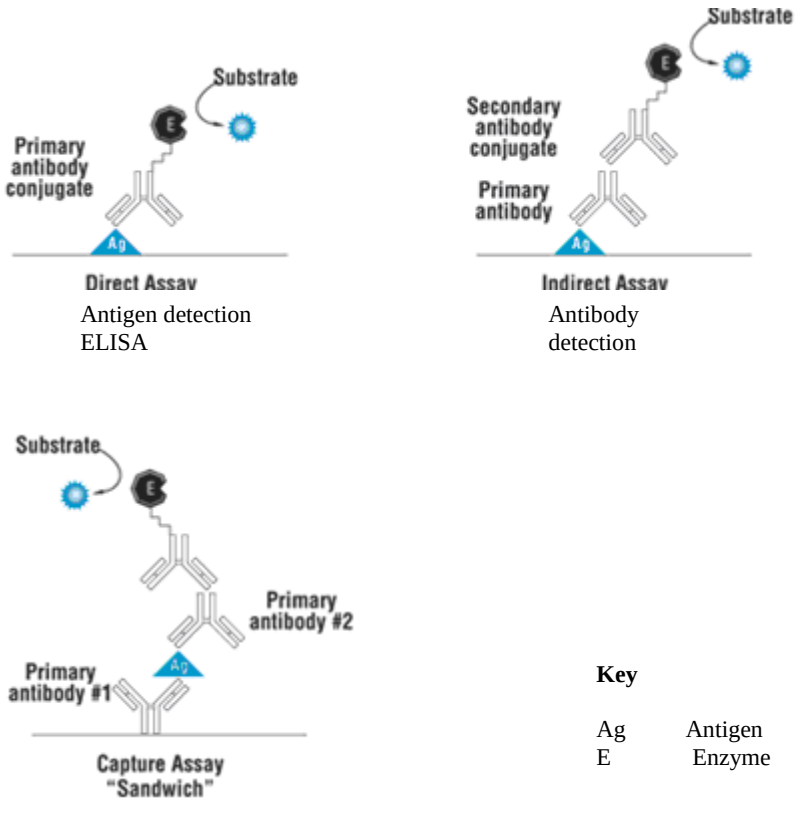
Figure 2.1 Structure of hepatitis B virus

HBV particle has an average diameter of 40nm, outer envelop made up of HBsAg proteins and an inner shell made up of capsid or core proteins enclosing a complex of DNA and viral polymerase

2.2 Enzyme Linked Immunosorbent Assays (ELISAs)

In 1969 Avrameas managed to couple two groups of proteins, antibodies and enzymes, by means of Glutaraldehyde Bridge (Avrameas and Ternynck, l969), a discovery that opened way for development of the first ELISA kit (Engvall *et al.,* 1971). In 1976, Organon Teknika Company developed and marketed the first commercially available ELISA system: the HBsAg ELISA system (Wolters *et al*., 1976). The principle of ELISA as described by Crowther (2001a) involves an analytical system in which an enzyme is used as a label on an antigen or antibody that binds to the antigen, antibody, immune complexes and sensitized T or B cells (analytes). After binding, the enzyme portion is assayed to allow for detection of an immune reaction and the estimation of analyte. ELISA systems are divided broadly into either homogeneous or heterogeneous formats

(Crowther, 2001a; Keith *et al.*, 2006). Homogeneous ELISA format does not require separation of reacted and non-reacted materials in order to detect or measure the target antigen while in heterogeneous assay format the bound and free analyte must be separated (Kurdziel *et al.*, 2001). There are three main methods, as described by Crowther (2001) that form the basis of both homogeneous and heterogeneous types of ELISA: Direct, Indirect, and Sandwich methods (Figure 2.2).



Adapted from Thermo Scientific Technical Information, (2007)

Figure 2.2 ELISA types

Three main types of ELISA that form the basis of both homogeneous and heterogeneous systems

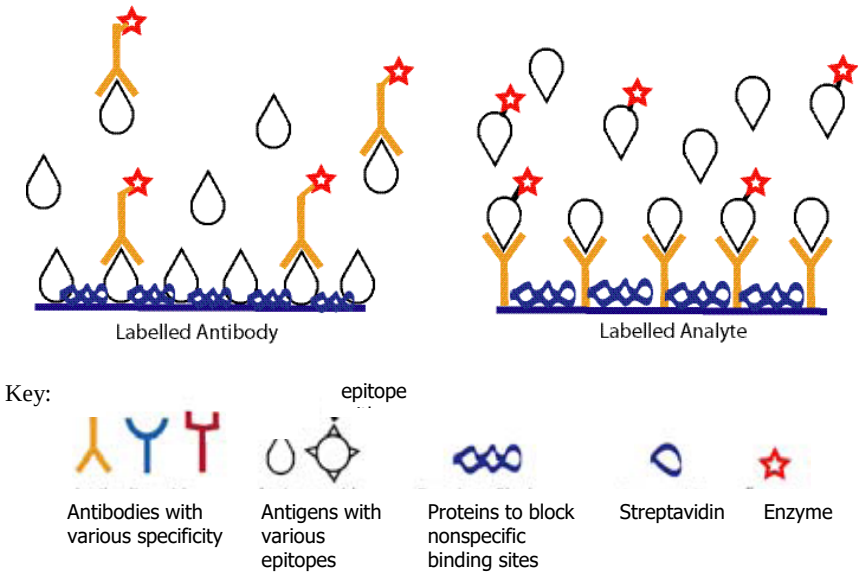
The direct ELISA as described by Levin (2005) uses the method of directly labeling the antibody. Microwell plates are coated with a sample containing the target antigen, and the binding of labeled antibody is quantified by a colorimetric, chemiluminescent, or fluorescent end-point. Since the secondary antibody step is omitted, the direct ELISA is relatively quick, and avoids potential problems of cross-reactivity of the secondary antibody with components in the antigen sample. However, the direct ELISA requires the labeling of every antibody to be used, which can be a time-consuming and expensive proposition (Crowther, 2001b). In addition, certain antibodies may be unsuitable for direct labeling. It also lacks additional signal amplification that can be achieved with the use of a secondary antibody (Crowther, 2001b).

The indirect ELISA is a two-step method that uses a labeled secondary antibody for detection (Jordan, 2005). A primary antibody is incubated with the antigen followed by incubation with a labeled secondary antibody that recognizes the primary antibody. A wide variety of labeled secondary antibodies are available commercially. The indirect ELISA system is versatile as many primary antibodies can be made in one species and the same labeled secondary antibody can be used for detection (Jordan, 2005). Immunoreactivity of the primary antibody is not affected by labeling and sensitivity is increased since each primary antibody contains several epitopes that can be bound by the labeled secondary antibody, allowing for signal amplification (Crowther, 2001b). Different visualization markers can be used with the same primary antibody.

The sandwich ELISA often referred to as “dual antibody sandwich (DAS)” or “dual antibody capture (DAC)” ELISA measures the amount of antigen between two layers of

antibodies (Jordan, 2005). The antigens to be measured must contain at least two antigenic sites, capable of binding to the antibody, since at least two antibodies act in the sandwich (Goldsby *et al.*, 2000). For this reason, sandwich assays are restricted to the quantitation of multivalent antigens such as proteins or polysaccharides (Crowther, 2001b). Sandwich ELISAs for quantitation of antigens are especially valuable when the concentration of antigens is low and/or they are contained in high concentrations of contaminating protein (Crowther, 2001b). The major advantages of this technique are that the antigen does not need to be purified prior to use, and that these assays are generally specific (Crowther, 2001b). However, one disadvantage is that not all antibodies can be used. Monoclonal antibody combinations must be qualified as "matched pairs", meaning that they can recognize separate epitopes on the antigen so they do not hinder each other's binding.

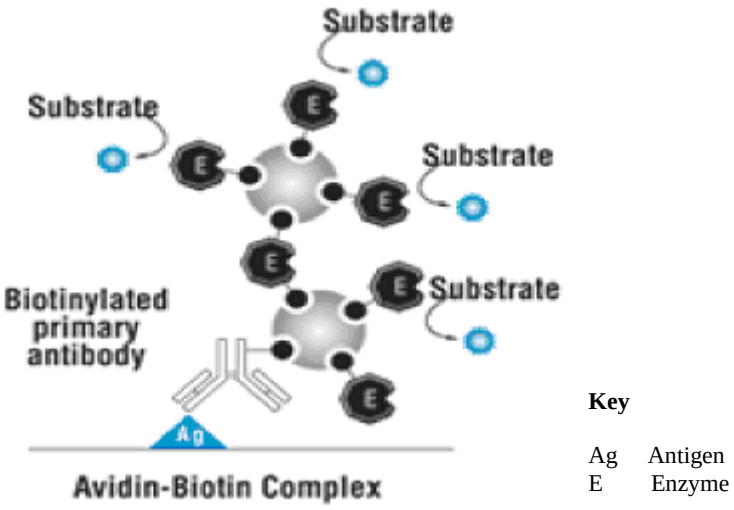
All the three ELISA systems, Direct, Indirect, and Sandwich methods, can be used in assays known as Competition or Inhibition ELISA (Crowther, 2001b). In Competitive methods one component of the immune reaction is immobilized and the other one labeled with an enzyme (Crowther, 2001b) (Figure 2.3). The analyte can then be quantified by its ability to prevent the formation of the complex between the immobilized and the labeled reagent. The advantages of this approach are that only one incubation step is necessary and that the "prozone effect" at high analyte concentrations does not occur (Voller *et al.*, 2005). The main disadvantages are that the concentration range in which the analyte can be quantified without sample dilution is rather narrow and that the antigen or antibody (in cases where either may be present in a sample produce the same response, and therefore cannot be distinguished in a one step assay (Yorde *et al.*, 1976).



Adapted from "Pierce Technical Guide for ELISA", (2007)

Figure 2.3 Competitive Assay Format

Another system of ELISA is the Biotin-Avidin ELISA system that can be directly-labelled, enzyme-tagged primary and secondary antibodies for detection of analytes.



Adapted from Thermo Scientific Technical Information

Figure 2.4 Avidin-Biotin ELISA system

The system as described by Jordan (2005) can generally achieve substantial amplification over the other methods due to the fact that many biotin molecules can be coupled to the antibody, enabling the biotinylated antibody to bind more than one molecule of avidin coupled to the enzyme *(*Figure 2.4*)*. As compared to the anti-HBs-HRP system the anti-HBs-biotin has an extra step that make the test takes longer time to carry out.

ELISA Reverse method and device, (Eberle and Seibl, 1992) is an ELISA technique that uses a solid phase made up of an immunosorbent polystyrene rod with 4-12 protruding ogives (curved shape). The entire device is immersed in a test tube containing the collected sample and then washed, incubated in conjugate and the in chromogen/substrate. The system has the advantage of allowing the simultaneous detection of different antibodies and antigens for multi-target assay (Eberle and Seibl, 1992). Also the use of laboratory supplies for dispensing sample aliquots, washing solution and reagents in microwells is not required hence facilitating ready-to-use lab-kits and on-site kits (Eberle and Seibl, 1992).

There is a relatively newer technique of ELISA known as Multiplex ELISA system (Virella *et al.,* 2007) that involves a protein array format that allows simultaneous detection of multiple analytes at multiple array within a single well. There are different types of Multiplex ELISAs that have been developed one of which measures antigens by coating or printing capture antibodies in an array format within a single well to allow for the construction of "sandwich" ELISA quantification assays (Virella *et al.,* 2007; Gonzalez *et al.,* 2008). Generally, Multiplex ELISA can also be achieved through antibody array, where different primary antibodies can be printed on glass plate to

capture corresponding antigens in a biological sample such as plasma, cell lysate, or tissue extract (Virella *et al.,* 2007). Detection method can be direct or indirect, sandwich or competitive, labeling or non-labeling, depending upon antibody array technologies (Imafuku *et al.,* 2004).

2.3 Development of the Sandwich ELISA Diagnostic Kits

2.3.1 Polyclonal and monoclonal antibodies

Polyclonal antibodies are derived from different B-cell lines and they are a mixture of immunoglobulin molecules secreted against a specific antigen, each recognizing a different epitope while monoclonal antibodies, on the other hands are derived from the same B-cell lines (Leland and Wu, 2005) hence monoclonal antibodies are more specific than polyclonal antibodies. Production of HBsAg polyclonal antibodies at KEMRI is achieved by using guinea pigs by the techniques described by Jackson and Fox. (1995). In this procedure guinea pigs are immunized with HBsAg that is extracted from HBV infected blood units and subsequently purified using rate zonal rotor ultracentrifuge and affinity chromatography. Blood units are harvested from the immunized guinea pigs after a period of three months and purified anti-HBs is obtained by Salt Immunoprecipitation and Affinity Chromatographic Techniques. Currently, these antibodies are used in manufacturing of a reverse passive hemoglutination (RPHA) based HBV screening kit, HEPCELL®, by the method described by Okoth *et al.* (1999). This kit has a diagnostic specificity and sensitivity of 98% and 99% respectively using an established monoclonal-based ELISA kit comparative "Gold" standard (Okoth *et al,* 1999) which is well within the acceptable limit for these parameters; sensitivity of 98% or more and a specificity of 95% or more (WHO/BCT/BTS/01.4, 2001). It is notable that majority of ELISA kits for

screening for HBsAg used in Kenya are manufactured from developed countries and are based on monoclonal antibodies that are slightly more specific, reproducible and purer but requiring a more elaborate and initially more financial capital to set up (Okoth *et al.*, 1999). A study by Anderson *et al.* (1983) found no difference in specificity and sensitivity between the adenovirus kits manufactured using a monoclonal antibody and one made using polyclonal antibody.

The main advantage of polyclonal antibodies over monoclonal antibodies is that they are able to detect variant forms of the antigens (Jongerius *et al*., 1997). In a study carried out by Louisirirotchanakul and Kanoksinsombat (2006), it was found commercial kits with monoclonal antibody capture and polyclonal antibody detection (mono/poly), but not mono/mono Ab capture and detection, could pick up the common HBsAg Gly145Arg mutant either solely or in combination with other mutations within the "a" determinant of HBsAg, which is the most vulnerable region for mutation.

Narhi and Caughey (1997) reported that the activity of the IgG used for coating the plate increases after denaturing with 50mM glycine-HCL at pH of 2.5 for 10 - 20 minutes at room temperature or at pH 7.0 in the presence of 6M urea or 6 M guanidine-HCl at pH 4.0 incubated overnight followed by dialysis into coating buffer. This is thought to be due to preferential denaturation of Fc regions that results in exposure of hydrophobic regions at the same time preserving the native conformation of the more stable Fab regions (Narhi and Caughey, 1997).

One of the biggest challenges in development of ELISA is the cross-activity of antibodies that occurs when an epitope is shared between the desired antigen and an irrelevant

antigen (KPL, 2008). Cross-reactivity due to similar epitopes are reduced by allowing longer incubation time that permit the reactants to come to the true equilibrium, increasing the concentration of salts or detergents and improving on the purification of the antigens (Thomson and Ketterhagen, 1984). To reduce interference due to the presence of anti-Ig antibodies, a nonspecific antibody from the species used to generate the antibody can be used (KPL, 2008). Monoclonal antibodies, contrary to the misconception that they are absolutely specific, can show some cross-reactivity as they can cross-react with epitopes having a similar three dimensional structure (KPL, 2008).

Concentration of polyclonal antibodies and other proteins in a solution can be estimated using the Absorbance Assay at 280nm or 200nm (Stoscheck, 1990). This assay operates on the principle that proteins in solution absorb ultraviolet light with absorbance maxima at 280nm and 200 nm. Amino acids with aromatic rings are the primary reason for the absorbance peak at 280 nm while peptide bonds are primarily responsible for the peak at 200 nm. Since secondary, tertiary, and quaternary structure protein can influence absorbance, factors such as pH and ionic strength of protein solution can alter the absorbance spectrum (Stoscheck, 1990). Antibody concentration can be calculated using following formula (Stoscheck, 1990).

$$\text{Concentration} = \frac{\text{Absorbance at 280 nm}}{\text{Extinction coefficient}}$$

Extinction coefficient - the factor by which the intensity of UV light decreases as it interacts with a unit thickness of an absorbing material. It is usually expressed per unit thickness

The path length of the UV light along the sample solution is 1 cm

Concentration is in mg/ml, %, or molarity depending on the type coefficient used:
The established absorbance coefficient of the IgG is 2.99 (Okoth *et al.*, 1999).

2.3.2. ELISA plates and adsorption of proteins

ELISA Plates adsorb proteins passively by hydrophobic interactions, van der Waals forces, hydrogen bonding and ionic interaction in order of increasing strength (Crowther, 2001b). In special cases where there is very little ability to form hydrophobic interaction such as when handling carbohydrates and heavily glycosylated protein, covalent linkages are used (Crowther, 2001b). According to Palfreyman *et al.* (1984), small peptides can be adsorbed on the plate but their epitopes are occasionally too few to be detected unless they are attached to large proteins through a spacer arm.

Adsorption of proteins on polystyrene surfaces takes place maximally at pH of or slightly above the isoelectric point (pI) as polystyrene surfaces are predominantly hydrophobic and at this pH there is minimal electrostatic repulsion (Crowther, 2001a). The most widely used coating buffers for antibodies are 50mM carbonate / bicarbonate (pH 9.6), 10mM Tris (pH 8.5) and 10mM PBS (pH 7.2) and contamination by detergents or/and extraneous proteins has to be avoided (Crowther, 2001b; Pierce, 2008).

When carrying out ELISA assay the "Edge Well Effects" must be considered. According to Esser (1995) the "Edge Well Effects" occur when ELISA results show differences in their absorbance values between edge wells and the wells in the central parts of the plate as a result of temperature variations between the middle and edges of the plate (as polystyrene is a poor conductor of heat) and also due to differential evaporation from the

wells. This phenomenon can be minimized by covering the plates during incubations and by ensuring that all the reagents are pre-equilibrated to the incubation temperature before their addition to the wells (Esser, 1995).

2.3.3 Blocking buffers and blocking of the void spaces on the ELISA plate

When ELISA plates are coated with proteins, some hydrophobic sites are left unoccupied. These sites need to be blocked in order to prevent non specific binding of subsequent reactants that could result in high background signal, low specificity and sensitivity (Zola, 1987; Crowther, 2001a). The blocking agents are mostly either detergent or proteins. Detergents, which have the disadvantage of blocking hydrophobic sites only, can belong to the ionic, zwitterionic and non-ionic classes. Non-ionic class in which Tween 20 and Triton X-100 belong, are the most widely used detergent blockers (Crowther, 2001a). Proteins such as Bovine Serum Albumin (BSA), Non-fat dry milk (NFDM), Normal serum, Casein or Caseinate and Fish gelatin are preferred as they can block both hydrophobic and hydrophilic sites of the plates and they can as well serve as a stabilizing agents preventing denaturation of the coated protein (Crowther, 2001a; Rosenberg, 2004). Another group of blockers is whole normal, or pre-immune serum that at times perform better than the earlier mentioned buffers in many immunoassays (Norland, 1986). This is due to the fact that normal serum contains a wide variety of biomolecules (proteins, glycoproteins, glycolipids) of various molecular weights and configurations such that hydrophobic, ionic, and covalent active sites on the surface can be blocked adequately. Fish serum and Mammalian serum, contains a wide variety of biomolecules and thus prevents nonspecific binding in the same efficient manner. However, as compared to mammalian serum, fish serum is a superior blocker due to the

fact that in evolutionary terms, the fishes are a distinct and remote group from mammals (Norland, 1986). Also, unlike mammalian sera, IgG (or similar antibody glycoproteins) are absent in fish serum. The major class of fish antibody is a tetrameric protein with a structure similar (but not cross-reactive) with the IgM pentamer of mammals. Therefore, fish serum shows little cross-reactivity with mammalian anti-IgG antibodies (Norland, 1986).

Sometimes combination of the blocking buffers such as the use of BSA and Tween 20 gives a better blocking property (Rosenberg, 2004). Optimization of the concentration of blocking buffer for a particular immunoassay is essential as using inadequate amounts of blocking buffer will results in excessive background and a reduced signal: noise ratio while using excessive concentrations of the same will mask antibody-antigen interactions or inhibit the enzyme hence causing a reduction of the signal: noise ratio (Crowther, 2001). There is no single blocking buffer that is ideal for every occasion as each antibody-antigen pair has unique characteristics (Crowther, 2001b).

2.3.4 Washing Buffers and Washing of the ELISA plates

Incubations of analytes in ELISA techniques generally results in high affinity specific interactions among the reactants and the excess reactants are diluted to undetected background levels by using washing buffers. Additionally, the interfering substances that are bound to the reactant with low affinity interactions are washed off (Crowther, 2001a). The essential element of the washing solution is a need to provide physiological buffer to avoid denaturation of the two binding reactants. The washing buffers commonly used are PBS, Tris saline and imidazole-buffered saline being commonly used together with

detergents such as Tween 20 and Triton X-100 at a concentration of 0.01 – 0.05% and sometimes with a protein such as BSA (Crowther, 2001b). The choice of washing buffer is generally dictated by other reagents in the ELISA. As an example, sodium azide and phosphate buffers are avoided when using HRP and alkaline phosphatase as the reporting enzymes respectfully (Emon *et al.*, 2007). To adequately dilute the excess reactants, it is of necessity that 3-5 washings are carried after each incubation and 5-10 minutes be preferably allowed before draining of the washing buffer to allow disruption of low affinity nonspecific interactions to come into the equilibrium. Insufficient washing results in high background signal while excessive washing may result in decreased sensitivity caused by elution of the antibody and / or antigen from the well (Crowther, 2001b).

2.3.5 Detection molecules in ELISA

The most widely used detection molecules are the enzymes horse radish peroxidase (HRP) and alkaline phosphatase (AP) as described by Goars (1983). Horse radish peroxidase, with molecular weight of 40 kDa, when lyophilized and stored at 4 ^{0}C is stable for a number of years and in solution its conjugate is stable for up to 1 year at 1.0mg/ml at 4 ^{0}C but its stability deteriorate at lower concentrations to as short as few weeks (Deshpande, 1996). To obtain the best stability of the HRP reagent and optimal sensitivity, according Deshpande (1996), it is important to use a two component substrate such as TMB and Hydrogen peroxide or Urea peroxide that are mixed prior to use. The two important chromogen substrate that serve as hydrogen donors for HRP are 3, 3', 5, 5' tetramethylbenzidine (TMB) and 2, 2-azino-di (3-ethyl-benzathiazoline) sulphonic acid (ABTS). Of the two TMB, which yields a blue coloured product with an absorbance at

650 nm and a yellow coloured product with an absorbance at 450nm in acidic media, is the most widely used (Diamandis and Christopoullus, 1996).

Antibody-HRP conjugates are superior to antibody-AP conjugates with respect to the specific activities of both the enzyme and antibody besides its provision of high turnover rate, good stability, low cost and wide availability of substrate (Crowther, 2001a). Alkaline Phosphatase catalysis the hydrolysis of phosphate group from the substrate molecule to produce coloured product and it has an advantages of giving reaction that remains linear whose detection sensitivity can be improved by allowing a reaction to proceed for a longer period (Crowther, 2001a)

2.3.6 Standardization and Optimization of the ELISA system

Calibration curves, as stipulated by Findlay (2006), form a critical component of preparation of ELISA kits and the use of at least six known concentrations of analytes is highly recommended. Jack (1995) recommends that samples dilutions that are to be used to prepare the calibration curve provide signals that fall in the vicinity of 50% B/B0 wherein B = y - b, B0 = y0 - b, y0 = the maximum signal and b is the selected point. Typically coefficients of variance (CV, standard deviation/mean) need to be less than 15% (Jack, 1995).

2.3.7 Checkerboard Titration

The target in developing an ELISA assay is to attain the best signal:noise ratio (defined as the signal generated by a sample containing analyte relative to the signal of the same

sample without analyte for the sensitivity level desired), to have a robust, reproducible assay for the sample being tested and to be able to measure the antigen over a biologically relevant assay range (dynamic range). Therefore, ideal concentrations of each assay reagent must be established empirically (Patton *et al*., 2005). One of the best known ways to determine the optimal concentration of various reagents such as capture, detection and conjugated antibodies in the development of ELISA kits, is by using Checkerboard titration techniques (Chart *et al.*, 1994; Pascho *et al.*, 1997; RD System, 2008). In this technique concentrations of two or more components are varied in a way that results in a pattern. One reagent (such as capture antibody) to be optimized is serially diluted and applied along the rows in the appropriate step of the procedure. The other reagent (such as detection antibody) to be optimized is serially diluted and applied along the columns also in the appropriate step of the procedure (Figure 2.5)

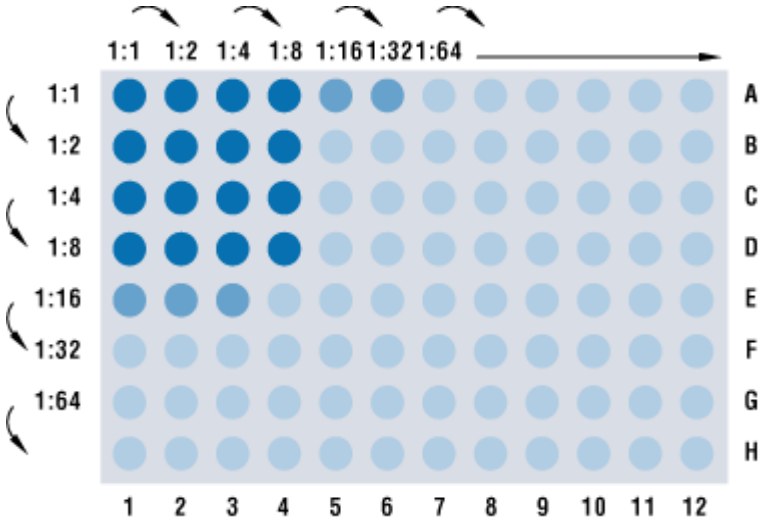


Figure 2.5 Checkerboard Titration Template

2.3.8 Cut-off points in ELISA systems

Cut-off points of tests are threshold or decision limits on the continuous scale of test results of which there are three main approaches of their determination (Jacobson, 1996). The first approach is a cut-off based on the frequency distributions of test results from uninfected and infected reference animals (Zweig and Campbell, 1993; Fawcet, 2006). A second, which is the one that is most widely used, is based only on uninfected reference animals (Greiner *et al.*, 1994). The third method provides an 'intrinsic cut-off' based on test results from sera drawn randomly from within the target population with no prior knowledge of the animals' infection status (Greiner *et al.*, 1994). When the initial stage of development of the ELISA is nearly complete Gao *et al.* (2004 and Jacobson (1996) advice that repeatability tests be undertaken. This is defined as agreement between replicates within and between runs of the assay and is necessary to warrant further development of the assay.

2.3.9 Analytical sensitivity and specificity in ELISA

The analytical sensitivity of the assay is the smallest detectable amount of the analyte in question, and analytical specificity is the degree to which the assay does not cross-react with other analytes (Crowther *et al*, 2001a). Analytical sensitivity is usally assessed in the laboratory by end-point dilution analysis, which indicates the dilution of serum in which antibody is no longer detectable (Crowther, 2001a). Internationally, the sensitivity of the Hepatitis diagnostic kits is determined by using the standard panel of sera obtained from Agence Française de Sécurité Sanitaire des Produits de Santé (AFSSaPS consisting of 9 samples with decreasing concentrations of HBsAg ranging from 2.22 to 0.06 ng/ml (Van

Roosmalen *et al.* 2006). Analytical specificity is assessed by use of a panel sera derived from animals that have experienced related infections that may stimulate cross-reactive antibodies (Jacobson, 1996).

2.3.10 Diagnostic Sensitivity and Diagnostic Specificity

Diagnostic sensitivity (D-SN) is the proportion of known infected reference animals that test positive in the assay while diagnostic specificity (D-SP) is the proportion of uninfected reference animals that test negative in the assay (Altman and Bland, 1994). In case of availability of relative standards of comparison only estimates of D-SN and D-SP for the new assay may be compromised because the error in the estimates of D-SN and D-SP for the relative standard is passed over into those estimates for the new assay (Jacobson, 2002). D-SN is the most important measure of a diagnostic method as it reflects the probability that a positive test reflects the underlying condition being tested for although its value does depend on the prevalence of the disease, which may vary (Gunnarsson *et al.*, 2002)

2.3.11 Maintenance of the validated state of ELISA kits

A validated assay needs constant monitoring and maintenance to retain that designation (Jacobson, 2002). Once the assay is put into routine use, internal quality control is accomplished by consistently monitoring the assay for assessment of repeatability and accuracy (Crowther, 2001). Stability test of the kit must also be carried out before the product is given the shelf life (Zheng *et al.*, 2005). Reproducibility between laboratories should be assessed at least twice each year (Falk *et al.*, 2000).

2.3.12 Pricing Techniques of the new kit

Most businesses fix their prices in accordance with the cost of production or provision as the price must cover these costs and contribute to the overall profitability of the business. The most common and easiest way of doing this is simply to add a fixed margin onto the cost price of the product (Cram, 2005: Shy, 2008)

CHAPTER THREE

3. MATERIALS AND METHODS

3.1 Laboratory materials, reagents and equipment

The following laboratory materials and equipment were used: ELISA Reader Multiscan (Labsystem, Finland), ELISA Reader ImmunoMini NJ-230 (Nalge-Nunc, Japan), Pipetman Gilson P10, P100, P200 and P1000 (France), Semillons, ELISA Plates 96 x .4ml Wells (SB Medical, Japan), UV Spectrophotometer (Shimadzu, Japan), Auto Mini Washer (Biotec, Japan), Magnetic Stirrer (Advantec, Japan), Electronic Balance GX-200 (A & D Company, Japan), Freezer, Medicool 4 ^{0}C (Sanyo, Japan), Plate Mixer (Biotec, Japan), Incubator (Advatec, Japan).

The following reagents and kits were used: Sodium carbonate (Fisher Scientific, UK), Sodium bicarbonate (Fisher Scientific, UK), Sodium azide (Fisher Scientific, UK), Tween 20 (Fisher Scientific, UK), Bovine serum albumin (Sigma-Aldrich, Germany), Disodium hydrogen phosphate (Fisher Scientific, UK), Pottasium dihydrogen phosphate (Fisher Scientific, UK), Sodium chloride (Fisher Scientific, UK), Sulphuric acid 98% pure (BDH, UK), Purified human HBsAg (KEMRI), Normal Human Serum (KEMRI), Ovine HRP conjugated anti-HBsAg antibodies (AbD Serotec); TMB (MP Biomedicals, France); Urea peroxide (Spectrum Chemicals, USA); HBV positive plasma (KEMRI), HEPCELL Reverse Passive Haemoglutination test kit (KEMRI), HEPCELL Rapid Test kit for HBV (KEMRI), Hepanostika HBsAg Ultra ELISA kit (Biomeriux, Germany), Guinea pig-produced anti-HBsAg (KEMRI), monoclonal anti-HBsAg (Immunon, USA), Lynx Rapid Conjugation Kits (AbD Serotec), Goat anti-HBsAg conjugate (AbD Serotec).

3.2 Preparation of reagents

All the reagents required for preparation of the coated ELISA plates and for carrying out the initial direct Sandwich ELISA test were prepared before the beginning of the subsequent steps as indicated in *Appendix 1*. These reagents included: Coating Buffer, Washing Buffer, Blocking Buffer, PBS, Stop Solution (0.5M Sulphuric acid), Positive Control, Negative Control and Conjugate. The appropriate reagents were weighed and dissolved in the appropriate media.

3.3 Coating the plate and carrying out the initial ELISA test

3.3.1 Assessing the binding capacity of the anti-HBs on the ELISA plate

Using the standard procedure described in the *Appendix 2*, an ELISA plate was coated with guinea pig (GP) generated HBsAg antibodies by 2-fold serial dilution along the columns starting with the initial concentration of 25µg (diluted from the stock solution of 306 µg/ml). The coated plate was blocked using 1% BSA and washing steps carried out using the Washing Buffer (0.05% Tween 20 in PBS). Approximately 0.44µg/ml of HBsAg positive plasma was placed in the whole of row A and serially diluted (double) through the whole of rows B and C. Approximately 18.92 µg/ml of the purified (neat) HBsAg was placed in the whole of row D and serially diluted (double) in rows E and F. The pooled negative control samples were placed uniformly in the whole of rows G and H. The ELISA procedure proceeded with the use of HRP-linked anti-HBs conjugate and TMB-Urea peroxide substrate. The ELISA plate was washed four times after both coating and blocking. It was also washed six times after incubating with conjugate. The numbers of washings have been used in other comparable procedures.

3.3.2 Assessment of the performance of various blocking agents against different lots of KEMRI generated Anti-HBs

The assessment of skimmed milk, BSA, NRS and NGPS blocking buffers was carried out using different batches of guinea-pig generated HBsAg polyclonal antibodies produced by KEMRI from 1994 to 2007 as follows: lots J-3, 99-8, and 37 (for plate 2a); lots 120499, 290699, and 36 (for plate 2b); lots 181201, 2, and 9901P (plate 2c). The design of the ELISA is shown in Tables 3.1, 3.2 and 3.3.

Table 3.1 Design of the ELISA – anti-HBs Lot no. J-3, 99-8 and 37

		J-3	99-8	37	J-3	99-8	37	J-3	99-8	37	J-3	99-8	37
		1.	**2.**	**3.**	**4.**	**5.**	**6.**	**7.**	**8.**	**9.**	**10.**	**11.**	**12.**
+Ve	**A**												
-Ve	**B**												
+Ve	**C**												
-Ve	**D**												
+Ve	**E**												
-Ve	**F**												
+Ve	**G**												
PBS	**H**												
		BSA 1%			**Skim 5%**			**NRS 0.3%**			**NGPS 0.3%**		

Table3.2 Design of the ELISA - anti-HBs Lot no. 120499, 290799 and 36

		120499	290799	36	120499	290799	36	120499	290799	36	120499	290799	36
		1.	**2.**	**3.**	**4.**	**5.**	**6.**	**7.**	**8.**	**9.**	**10.**	**11.**	**12.**
+Ve	**A**												
-Ve	**B**												
+Ve	**C**												
-Ve	**D**												
+Ve	**E**												
-Ve	**F**												
+Ve	**G**												
PBS	**H**												
		BSA 1%			**Skim 5%**			**NRS 0.3%**			**NGPS 0.3%**		

Table 3.3 Design of the ELISA - anti-HBs Lot no. 181201, 2 and 9901P

		181201	2	9901P	181201	2	9901P	181201	2	9901P	181201	2	9901P
		1.	**2.**	**3.**	**4.**	**5.**	**6.**	**7.**	**8.**	**9.**	**10.**	**11.**	**12.**
+Ve	**A**												
-Ve	**B**												
+Ve	**C**												
-Ve	**D**												
+Ve	**E**												
-Ve	**F**												
+Ve	**G**												
PBS	**H**												
		BSA 1%			**Skim 5%**			**NRS 0.3%**			**NGPS 0.3%**		

3.3.3 Selection of conjugate, TMB substrate and determination of Cut-off values

The effect of using different conjugates on the performance of the ELISA kit was determined by coating the ELISA plate with 5µg /ml of anti-HBs, blocking the coated plate with BSAT and applying 2-fold serially diluted purified HBsAg along the 4 rows of the plate. Different HRP-linked anti-HBs conjugates were applied to each row as follows: goat (laboratory prepared), rabbit, mouse (monoclonal) and goat (commercial). The ELISA was run as described in *Appendix 3*.

To assess the performance of ovine and goat HRP-linked anti-Hbs conjugates in BSAT blocking buffer, an ELISA was run using the following parameters: 5µg /ml of coating anti-HBs; 1% bovine serum albumin and 0.05% solution (BSAT) blocking buffer; 2-fold dilutions of purified HBsAg; goat HRP-linked anti-HBs conjugate (duplicate); ovine HRP-linked anti-HBs conjugate (duplicate) and TMB-Urea Peroxidase substrate. The ELISA was run as described in *Appendix 3*.

To compare the performance of the TMB-Urea peroxide and TMB-Hydrogen peroxide ELISA system, an ELISA was run using coating anti-HBs (5µg /ml), blocking buffer (BSAT), conjugate (ovine HRP-linked anti-HBs) and substrates (TMB-Urea peroxide and TMB-Hydrogen peroxide in duplicate). The ELISA was run as described in *Appendix 3* Provisional cut-off point was determined as per the technique described by Greiner *et al.* (1994). The pooled negative samples were loaded into 11 wells of row D of the plate and the 12th well of the same row was loaded with diluent only. The pooled positive control sample was equally loaded in 11 wells of row E and the 12th well of the same row was loaded with sample diluent only. ELISA test was then performed up to the last step of reading the absorbance. Provisional cut-off was calculated using the mean absorbance values plus 0.2 (Greiner *et al.*, 1994).

3.3.4 Optimization of capture and detection antibody concentrations through the checkerboard titrations

The optimal concentrations of the capture and detection conjugated antibodies in the development of ELISA kit were determined by the simple checkerboard titration technique as shown (Figure 2.5) and fully described by Chart and Frost (1994) and Pascho *et al.* (1997). In this technique, the capture antibody was serially diluted and applied along the rows starting with concentration of 10µg/ml and the ovine HRP-anti-HBs conjugate was serially diluted starting with the dilution of 1:600 and applied along the column at the appropriate stage of the ELISA procedure.

3.3.5 Determination of analytical sensitivity and specificity

The working standard of the purified HBsAg was prepared using spectrophotometric absorbance technique described by Stoscheck (1990). Briefly a stock solution of affinity-purified HBsAg prepared at KEMRI Production Department following method described by Okoth *et al.* (1999) was taken. Three readings of the absorbance values of this solution were read using UV spectrophotometer (Shimadzu, Japan) and the mean OD value was calculated.

The following formula (Stoscheck, 1990) was used to calculate the concentration of the purified HBsAg:

Concentration of HBsAg = Mean Absorbance Value / 2.99 mg per ml

Where 2.99 is the extinction coefficient of HBsAg (the factor by which the intensity of UV light decreases as it interacts with a unit thickness of HBsAg solution)

Analytical sensitivity was assessed by end-point dilution analysis techniques described by Crowther (2001). In this study purified HBsAg positive sample was applied on the first well of the row and serially diluting it along the row upto the 12^{th} well. The ELISA procedure proceeded with the use of HRP-linked anti-HBs conjugate and TMB-Urea peroxide substrate at the appropriate stage. Since the HBsAg positive sample could not be standardized using internationally standardized HBsAg positive sera from Agence Française de Sécurité Sanitaire des Produits de Santé (AFSSaPS) as reported by Van Roosmalen (2006), the same procedure was repeated with Hepanostika HBsAg Utra® (Biomerioux) whose analytical sensitivity had been determined to be 0.12ng/ml (Van Roosmalen *et al*, 2006). By comparing the values of the two tests it was possible to get

an estimated analytical sensitivity of the developed ELISA kit. Analytical specificity was assessed by running the ELISA of the developed kit against the panel sera of HIV and Hepatitis C viruses

3.4 Diagnostic sensitivity and specificity

A set of 96 samples of panel plasma/sera (72 plasma and 24 sera) were picked for determination of diagnostic sensitivity and specificity.

These samples were analyzed using the kit developed in this project, HEPCELL® PHA, HEPCELL® Rapid and HEPANOSTIKA HBsAg Ultra® kits to determine comparative performance. The HEPANOSTIKA HBsAg Ultra® kit was used as the comparative Gold Standard. The 2 x 2 Contingency table was prepared to help in calculation of diagnostic sensitivity and specificity using the following formula described by Crowther, (2001a):

$$\text{Diagnostic Sensitivity \%} = \frac{\text{True positives (TP)} \times 100}{\text{True positives (TP)} + \text{False Negatives (FN)}}$$

$$\text{Diagnostic Specificity \%} = \frac{\text{True Negatives (TN)} \times 100}{\text{False Positives (FP)} + \text{True Negatives (TN)}}$$

$$\text{Positive Predictive Value \%} = \frac{\text{True positives (TP)} \times 100}{\text{True positives (TP)} + \text{False positives (TP)}}$$

$$\text{Negative Predictive Value \%} = \frac{\text{True negative (TN)} \times 100}{\text{True Negatives (TN)} + \text{False negative (FN)}}$$

3.5 Repeatability and reproducibility

Repeatability analysis in the diagnostic assay was done using the technique described by Gao *et al* (2004) and Jacobson (1996) where two elements are involved: the amount of agreement between two replicates of each sample within a run of the assay, and the amount of agreement between runs for the normalized values of each control sample. Three replicates of each sample were run with five plates on five separate occasions. Coefficients of variation (standard deviation of replicates/mean of replicates) were calculated and it was expected to be less than 20% for raw absorbance values (Reed *et al.,* 2002). Inter-subject variability was also determined by giving two analysts in the KEMRI Production Department Laboratory the developed kit to carry out similar tests.

3.6 Costing of the new HBsAg detection ELISA kit

The costing of the developed HBsAg detection ELISA was calculated using the formula described by Shy (2008) and the computation is shown *(Appendix 6).*

3.7 Monitoring Validity of Assay Performance

The stability of the developed ELISA kit would be monitored on continuous basis long after the end of this project.

3.8 Prices search for HBsAg detection kits in Kenya

Prices of the most widely used HBsAg detection kit in Kenya were obtained from one of the major distributors of the diagnostic kits in East Africa – HASS SCIENTIFIC LTD, Nairobi, Kenya.

CHAPTER FOUR

4 RESULTS

4.1 The initial stages of preparation of the ELISA kit

4.1.1 Binding of the anti-HBs on the ELISA plate

Wells that were coated with anti-HBs and samples of purified HBsAg, HBsAg positive plasma and pooled negative controls applied separately yielded the results shown by Plate 4.1 and Table 4.1. The intensity of the developed colour when visually observed decreased along the rows for the three samples. Wells where purified HBsAg had been applied showed the deepest colour intensity which was an expected outcome due to higher concentration of HBsAg, as compared to the in HBsAg positive plasma. Wells were getting clearer with the reduced concentrations of coated anti-HBs indicating that the BSA was effective in blocking the sites unoccupied by anti-HBs.

Figure 4.1 shows that the purified HBsAg samples had the highest optical density values followed by the HBsAg positive plasma as expected. The background signals (optical densities) shown by the HBsAg negative sample were generally high and it decreased with the decreasing concentration of the capture anti-HBs implying that the elements causing these nonspecific reactions were closely associated with the coated anti-HBs. The two rows in which the HBsAg negative samples were applied (G & H) had closely similar patterns of the variation of optical density indicating good reproducibility profile of the test (Correlation of coefficient, r, 0.99). The p-values for rows G and H were 0.91 and 0.92 respectively at $\alpha \leq 0.05$)

In the rows where the purified HBsAg was applied the intensity of the signal decreased with the decreasing concentrations of the anti-HBs with a very notable drop of the gradient from anti-HBs concentration of 1.56µg/ml to 0.2µg/ml. This indicates that wells of the ELISA plate were saturated with the coated anti-HBs at concentrations higher than 1.56µg/ml. The quantity of anti-HBs bound on the plate at the concentration below 0.2µg/ml was too little to be detected.

In the rows where the HBsAg positive plasma sample was applied (A, B and C) the pattern of variation of the absorbance with dilution of sample was such that the absorbance of undiluted sample was highest but the second highest dilution had the lowest optical density. This may have been due to experimental errors.

The results of this experiment were used to make the decision of using 5µg/ml of anti-HBs for coating ELISA plates in subsequent experiments. This was in accordance to the recommendation by Crowther (2001) that the concentration used for coating be at the stage where the plateau had stabilized.

Plate 4.1 ELISA results demonstrating the coating potential of anti-HBs to the ELISA plate

The 2-fold serially diluted anti-HBs were coated along the columns, starting with 25µg/ml. The 2-fold serially diluted samples were applied along the rows as follows: HBsAg +ve plasma (A to C) and purified HBsAg (D to F). HBsAg -ve sample was applied uniformly in rows G and H.

1 2 3 4 5 6 7 8 9 10 11 12

A

B

C

D

E

F

G

H

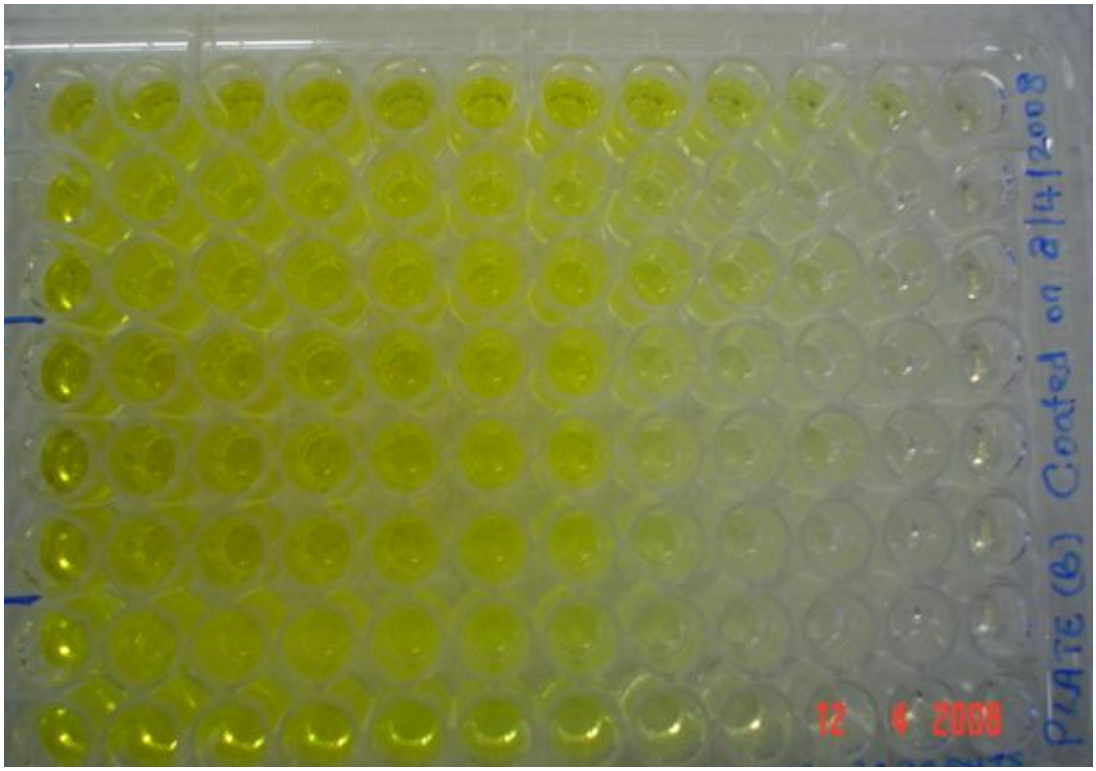


Table 4.1 Optical densities of the ELISA to demonstrate the coating potential of anti-HBs

The 2-fold serially diluted anti-HBs were coated along the columns, starting with 25µg/ml. The 2-fold serially diluted samples were applied along the rows as follows: HBsAg +ve plasma (A to C) and purified HBsAg (D to F). HBsAg -ve sample was applied uniformly in rows G and H.

	ELISA plate column numbers												
ELISA plate row numbers		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
	A	1.27	1.12	1.13	1.11	0.97	0.93	0.71	0.42	0.21	0.12	0.06	0.01
	B	0.80	0.78	0.71	0.67	0.55	0.44	0.30	0.17	0.08	0.03	0.00	0.01
	C	1.19	1.15	1.01	1.09	0.92	0.81	0.51	0.29	0.14	0.07	0.03	0.01
	D	1.66	1.58	1.52	1.49	1.38	1.07	0.53	0.16	0.07	0.02	0.01	0.01
	E	1.58	1.49	1.43	1.42	1.33	1.05	0.48	0.15	0.05	0.08	0.02	0.01
	F	1.46	1.37	1.37	1.33	1.24	0.88	0.33	0.14	0.05	0.02	0.00	0.00
	G	0.61	0.66	0.61	0.55	0.43	0.33	0.21	0.10	0.04	0.02	0.00	0.01
	H	0.58	0.62	0.58	0.53	0.40	0.33	0.20	0.10	0.03	0.01	0.01	0.02

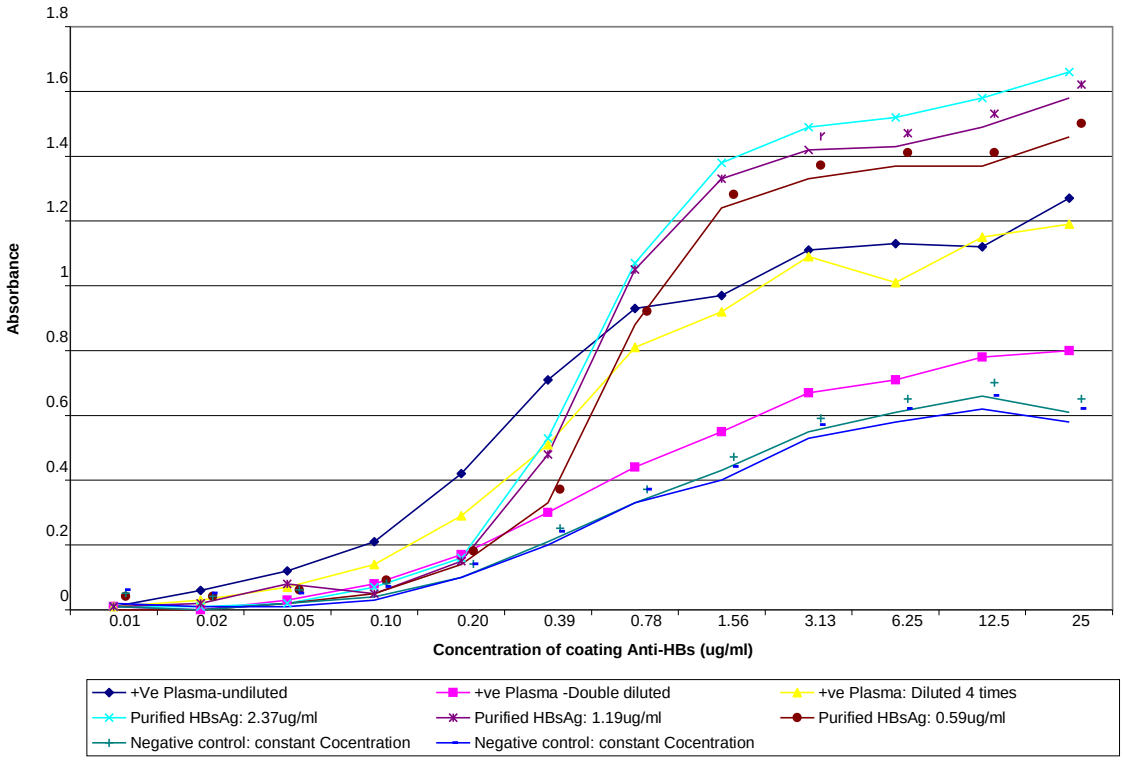


Figure 4.1 Patterns of ODs of various samples demonstrating coating potential of anti-HBs

The 2-fold serially diluted anti-HBs were coated along the columns, starting with 25µg/ml. The 2-fold serially diluted samples were applied along the rows. Correlation of coefficient, r, of wells where negative control was applied was 0.99). The p-values for rows G and H were 0.91 and 0.92 respectively at $\alpha \leq 0.05$)

4.1.2 Performance of various blockers against different lots of Anti-HBs

The assessment of skimmed milk, bovine serum albumin (BSA), normal rabbit serum (NRS) and normal guinea pig serum (NGPS) blocking buffers was carried out using various lots of anti-HBs to obtain the results shown in Plate 4.2a, 4.2b and 4.2c and Tables 4.2a, 4.2b and 4.2c. From the visual appearance of the Plate 4.2a, 4.2b and 4.2c and ODs given in Tables 4.2a, 4.2b and 4.2c, the wells blocked with skimmed milk gave

the weakest signal with HBsAg positive samples, an observation that is confirmed in Figure 4.2. This implies that the skimmed milk was suppressing signal from HBsAg positive samples besides blocking spaces unoccupied by coating anti-HBs.

Wells blocked with 1% BSA showed the highest signal of 1.321 for the HBsAg positive samples, HBsAg negative samples, and the blanks as shown in Figures 4.2 but they at the same time showed low Signal: Noise ratio (3) as shown in Figures 4.3. Wells blocked with the skimmed milk had the lowest signal (0.218) for HBsAg positive samples as seen in Figures 4.2. The Ratios of mean absorbance of HBsAg negative plasma sample to the corresponding blank value was generally highest in wells blocked with 1% BSA for all lots anti-HBs (Figure 4.3). Generally wells blocked with 0.3% Normal Rabbit Serum (NRS) and 0.3% Normal Guinea Pig Serum (NGPS) showed the highest Signal: Noise Ratio with the former showing the highest values of 18. This implied that the two buffers were not suppressing the signal from HBsAg positive samples and they were effective in blocking the spaces unoccupied by the coating anti-HBs. There was no clear trend that demonstrated superiority of any anti-HBs produced by KEMRI.

The results of this experiment and the availability of BSA and NRS were used to make the decision of using 1% BSA and 0.3% NRS as blocking buffers in the subsequent experiment.

Plates 4.2a, 2b and 2c: Performance of various blocking agents against different lots of anti-HBs

Three ELISA plates were coated with 5ug/ml of different lots of anti-HBs and the blocking buffers applied in columns of each plate as follows: BSA (1-3), skimmed milk (4-6), NRS (7-9) and NGS (10-12) as illustrated in Tables 3-1, 3-2 and 3-3. Wells blocked with skimmed showed the lowest colour intensity.

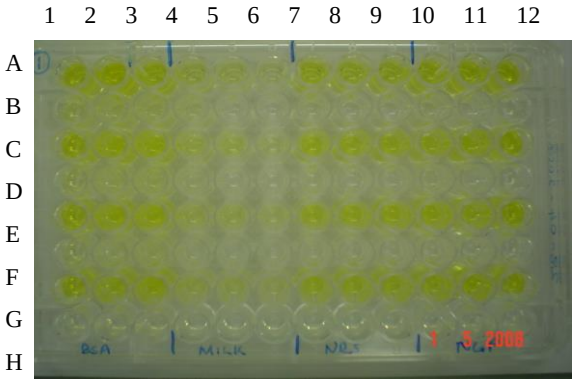


Plate 4.2a *(Anti-HBs lot nos.J-3, 99-8, 37)*

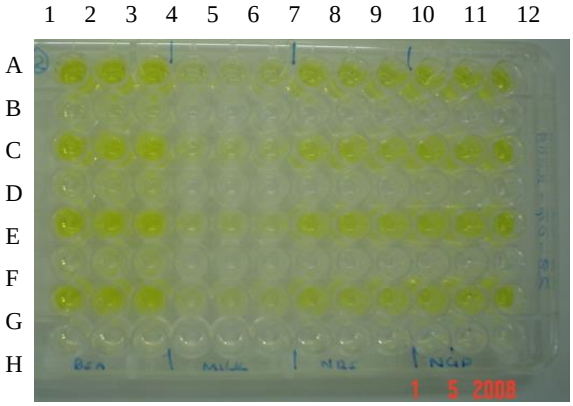


Plate 4.2b *(Anti-HBs lot nos.120499, 290799, 36)*

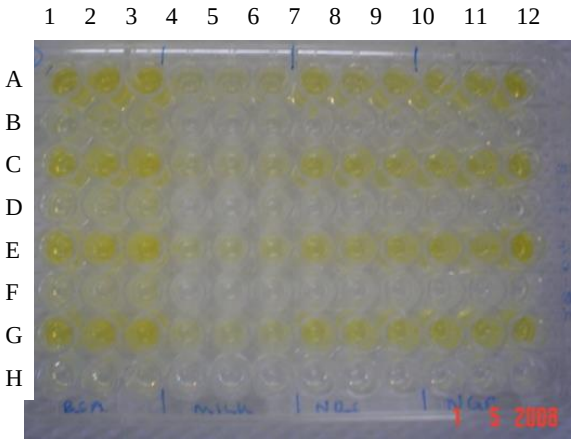


Plate 4.2c *(Anti-HBs lot nos.181201, 2, 9901P)*

Tables 4.2a, 2b and 2c: Performance of various blocking agents against different lots of anti-HBs

Three ELISA plates were coated with 5ug/ml of different lots of anti-HBs and the blocking buffers applied in columns of each plate as follows: BSA (1-3), skimmed milk (4-6), NRS (7-9) and NGS (10-12) as illustrated in Tables 4-2a, 4-2b & 4-2c.

Table 4-2a *(J-3, 99-8, 37)*

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.199	1.84	1.055	0.406	0.301	0.191	0.768	0.613	0.653	0.847	0.906	0.892
B	0.23	0.216	0.21	0.15	0.053	0.042	0.093	0.046	0.042	0.095	0.043	0.045
C	0.995	1.15	1.034	0.34	0.323	0.189	0.777	0.699	0.763	0.73	0.857	0.904
D	0.195	0.211	0.21	0.111	0.055	0.053	0.083	0.044	0.051	0.089	0.05	0.046
E	0.947	1.026	0.999	0.312	0.307	0.218	0.677	0.719	0.799	0.864	0.712	0.928
F	0.161	0.155	0.198	0.079	0.053	0.062	0.071	0.051	0.044	0.061	0.049	0.056
G	0.878	0.87	1.104	0.339	0.321	0.251	0.731	0.734	0.766	0.788	0.958	0.819
H	0.076	0.079	0.073	0.057	0.045	0.049	0.048	0.048	0.051	0.049	0.054	0.05

Table 4-2b *(120499, 290799, 36)*

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.495	1.369	1.384	0.264	0.223	0.346	0.702	0.577	0.59	0.765	0.72	0.702
B	0.15	0.188	0.193	0.047	0.05	0.054	0.047	0.049	0.046	0.052	0.054	0.047
C	1.149	1.223	1.226	0.248	0.255	0.227	0.623	0.664	0.571	0.816	0.736	0.791
D	0.182	0.211	0.234	0.049	0.057	0.052	0.046	0.047	0.048	0.082	0.061	0.065
E	1.163	1.248	1.248	0.253	0.26	0.233	0.684	0.726	0.657	0.769	0.801	0.865
F	0.153	0.21	0.212	0.055	0.058	0.06	0.05	0.046	0.052	0.046	0.046	0.053
G	1.161	1.231	1.273	0.215	0.265	0.271	0.65	0.562	0.589	0.696	0.654	0.841
H	0.089	0.088	0.083	0.055	0.052	0.053	0.055	0.054	0.052	0.047	0.046	0.059

Table 4-2c *(181201, 2, 9901P*

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.273	1.531	1.47	0.285	0.274	0.328	0.769	0.489	0.631	0.668	0.68	1.006
B	0.181	0.286	0.275	0.048	0.062	0.097	0.049	0.057	0.058	0.104	0.06	0.06
C	1.068	1.185	1.413	0.257	0.253	0.3	0.741	0.721	0.65	0.685	0.745	0.804
D	0.244	0.241	0.285	0.044	0.065	0.118	0.053	0.043	0.082	0.069	0.049	0.073
E	1.144	1.301	1.476	0.279	0.264	0.353	0.608	0.534	0.669	0.819	0.625	1.321
F	0.235	0.207	0.266	0.054	0.067	0.079	0.043	0.059	0.059	0.053	0.059	0.064
G	1.016	1.24	1.437	0.275	0.283	0.309	0.743	0.672	0.693	0.842	0.705	0.775
H	0.109	0.108	0.105	0.052	0.046	0.046	0.046	0.052	0.046	0.05	0.055	0.051

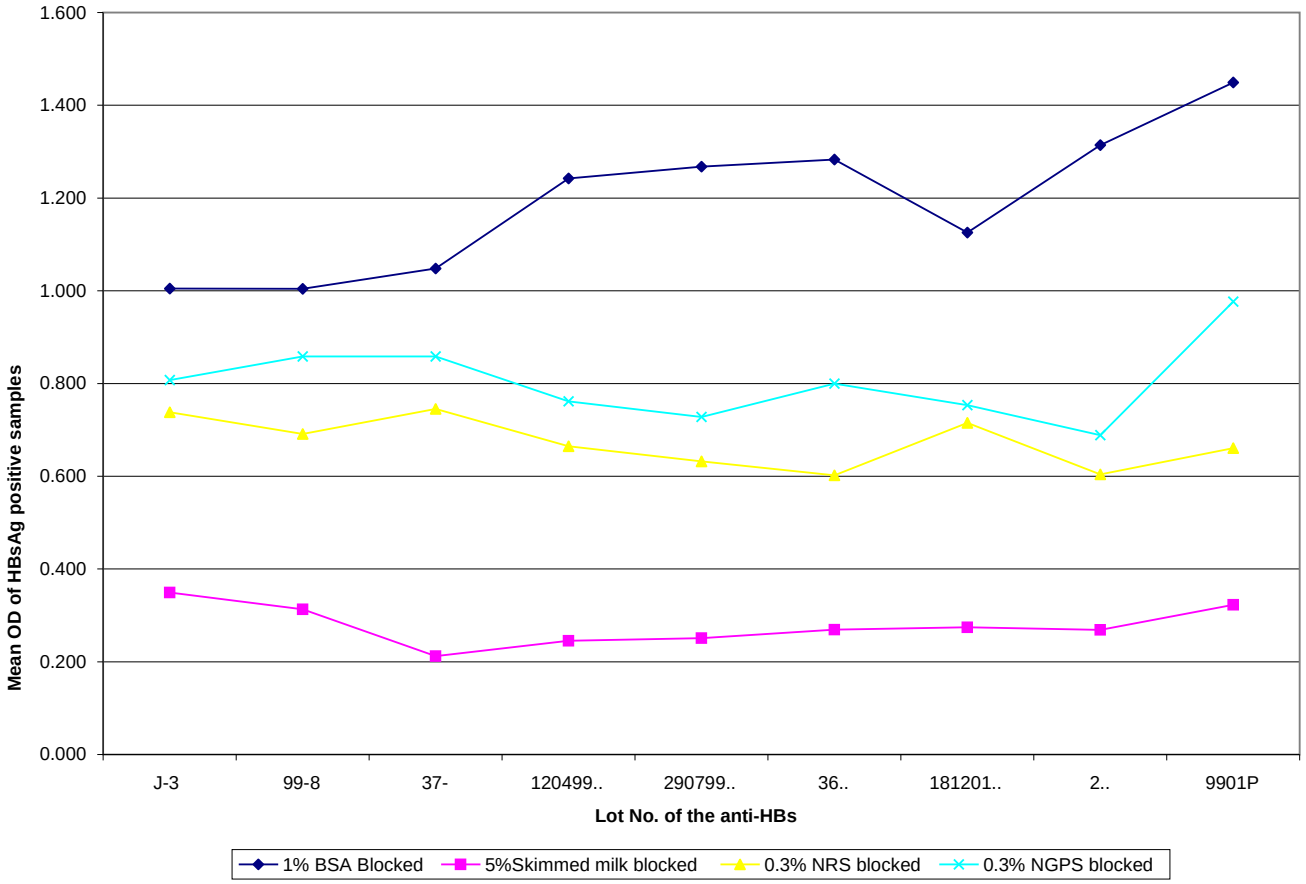


Figure 4.2 Performance of capture anti-HBs and blocking buffers (HBsAg +ve samples)

Three ELISA plates were coated with 5ug/ml of different lots of anti-HBs and the blocking buffers applied in columns of each plate as follows: BSA (1-3), skimmed milk (4-6), NRS (7-9) and NGS (10-12). P-values: BSA – 0.989; skimmed milk – 0.920; NRS – 0.894 and NGS- 0.940

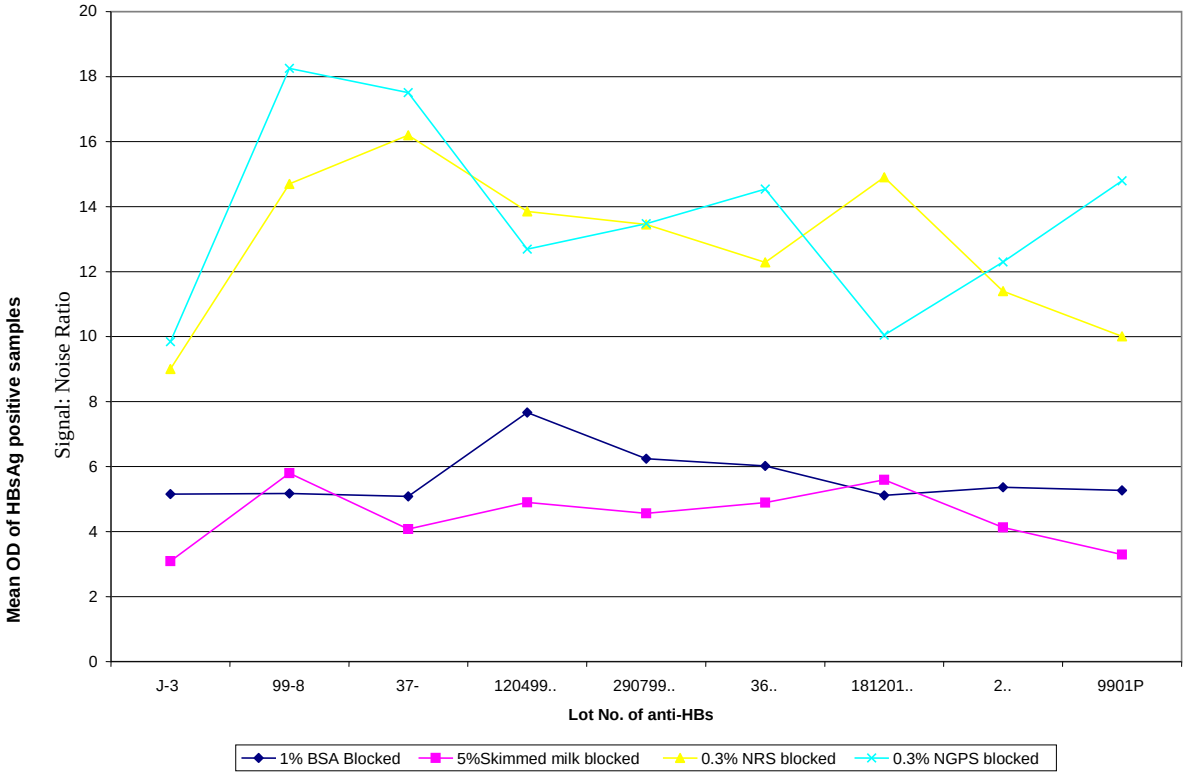


Figure 4.3 Performance of anti-HBs and blocking buffers in respect to the signal: Noise Ratio

Three ELISA plates were coated with 5ug/ml of different lots of anti-HBs and the blocking buffers applied in columns of each plate as follows: BSA (1-3), skimmed milk (4-6), NRS (7-9) and NGS (10-12). The mean ODs of HBsAg positive plasma sample obtained were divided by the corresponding mean ODs for the negative plasma samples and the values obtained were used to construct the graph below. P-values: BSA - 0.99; Skimmed milk - 0.92; NRS - 0.91 and NGPS - 0.860

4.1.3 Comparison of the performance of 1% BSA and 1% BSA in 0.05% Tween 20 (BSAT) as blocking buffers

The experiment was carried out to compare the performance of 1% BSA and 1% BSA in 0.05% Tween 20 (BSAT) as blocking buffers. The performance of 1% BSA had been described in section 4.1.2 and 4.1.3. Plate 4.3 and Table 4.3 showed clear distinction between wells where HBsAg positive plasma was applied (yellow colour) in comparison to the wells where HBsAg negative plasma and the normal human serum were applied (clear). Wells blocked with BSAT showed higher signals (3 times) than wells blocked with BSA after the application of HBsAg positive plasma sample, apart from column 10 where the absorbance was low (Figure 4.4), possibly due to accidental contamination with the HBsAg positive sample. The observation of higher absorbance values in wells blocked with BSAT support the observation by Rosenberg (2004) that sometimes BSAT is a better blocker than BSA. Row G was coated with appropriate anti-HBs and not blocked; unexpectedly the background signal was not elevated. Row H was not coated with anti-HBs but was blocked with 1 % BSA; again, unexpectedly, the OD was high where a HBsAg positive sample was applied. These two incidences required further follow up study. Both anti-HBS lots (99-8 and 120799) showed close patterns of optical densities in wells blocked with 1% BSA.

The results of this experiment were used to make the decision of using 1%/0.05% BSAT as the blocking buffer in subsequent experiments in the study.

Plate 4.3 Assessment of the BSA and BSAT blocking buffers

Column 1 to 6 of rows E, F and G were coated with anti-HBs lot # 99-8 while columns 7 to 12 of the same rows were coated with anti-HBs lot #120799. Row H was not coated with any antibody. 1% BSA Blocking buffers was applied in row E and H and 1% / 0.005% BSAT blocking buffer was applied in row F. Samples were applied as follows: HBsAg +ve plasma: columns 1, 4, 7 and 10; HBsAg -ve plasma in columns 2, 5, 8 and 11.

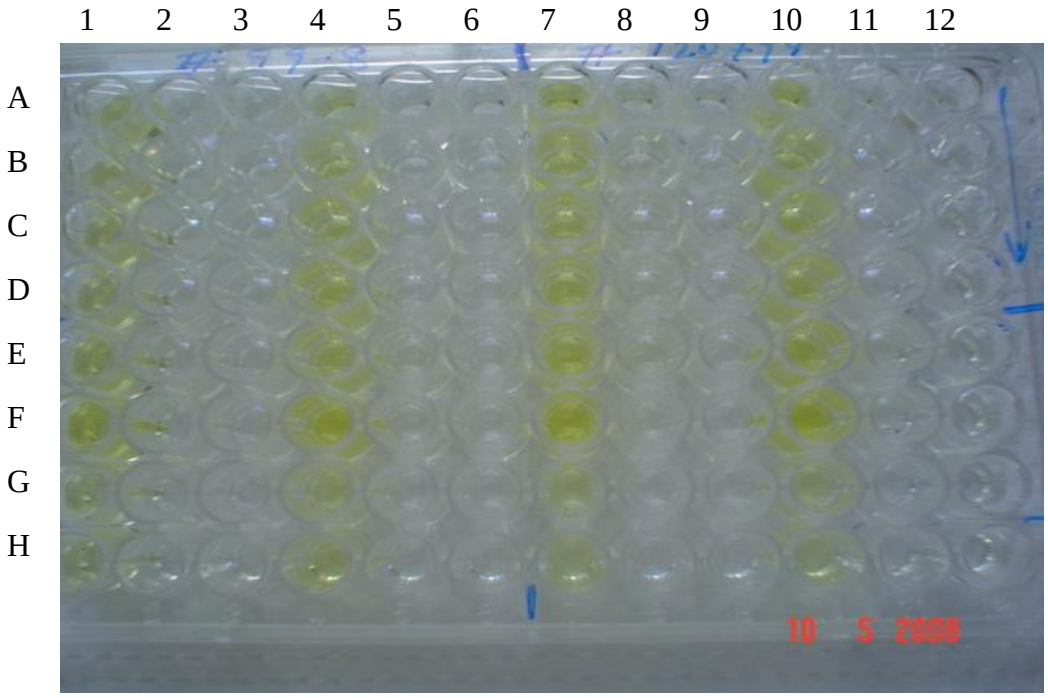


Table 4.3 The ODs of the wells used for assessment of the BSA and BSAT blocking buffers

Column 1 to 6 of rows E, F and G were coated with anti-HBs lot # 99-8 while columns 7 to 12 of the same rows were coated with anti-HBs lot #120799. Row H was not coated with any antibody. 1% BSA Blocking buffers was applied in row E and H and 1% / 0.005% BSAT blocking buffer was applied in row F. Samples were applied as follows: HBsAg +ve plasma: columns 1, 4, 7 and 10; HBsAg -ve plasma in columns 2, 5, 8 and 11.

		ELISA plate column numbers											
		1.	**2.**	**3.**	**4.**	**5.**	**6.**	**7.**	**8.**	**9.**	**10.**	**11.**	**12.**
ELISA plate rows	**E**	0.217	0.057	0.044	0.293	0.065	0.05	0.23	0.062	0.048	0.261	0.07	0.05
	F	0.702	0.066	0.061	0.824	0.071	0.054	0.983	0.073	0.056	0.05	0.07	0.06
	G	0.17	0.055	0.047	0.186	0.051	0.045	0.197	0.097	0.048	0.195	0.07	0.05
	H	0.209	0.053	0.048	0.2	0.051	0.049	0.218	0.061	0.058	0.203	0.06	0.05

4.1.4 Selection of Conjugate

As shown in Figure 4.5 the goat anti-HBs conjugate acquired commercially, showed the highest signal with HBsAg positive sample albeit with a slightly elevated background signal. The trend of the graph for this particular conjugate was less regular as compared to the others, possibly due to the experimental errors. The three HRP-linked anti-HBS conjugates prepared in-house by conjugation of goat anti-HBs, rabbit anti-HBs and monoclonal anti-HBs with the commercially acquired conjugation kit generally showed lower signals (Figure 4.5).

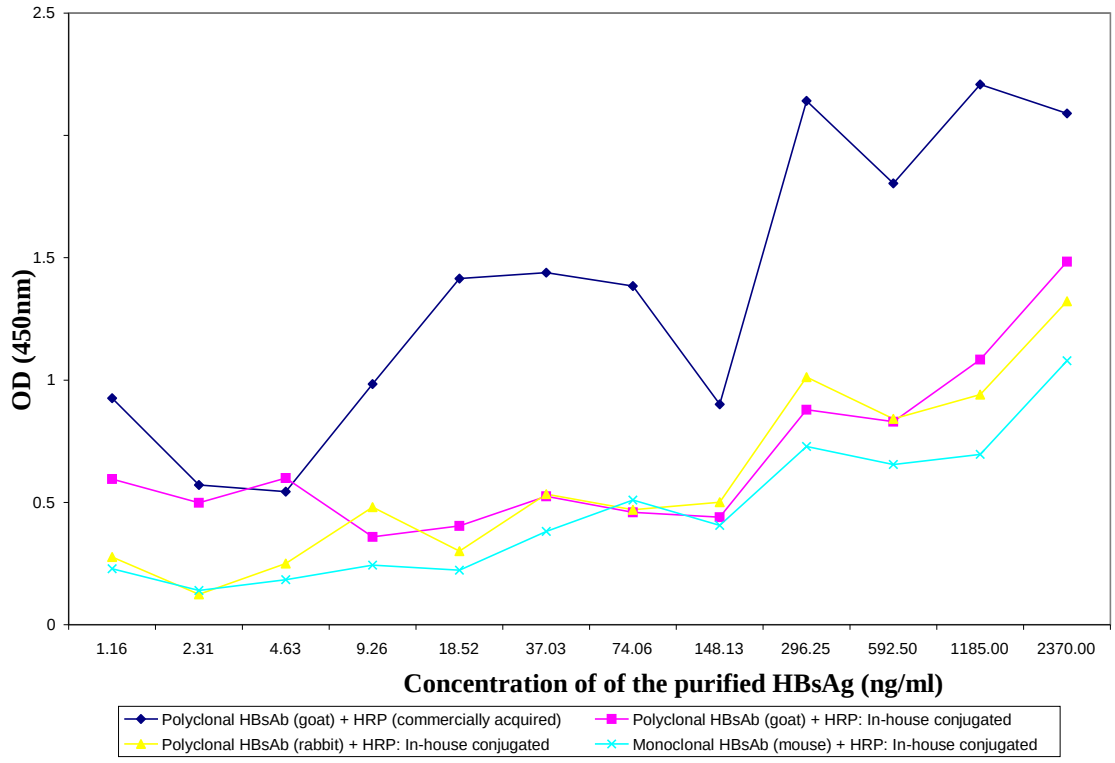


Figure 4.4 Effect of using different conjugates on the performance of the ELISA kit

The plate was coated with 5ug/ml, blocked with BSAT and purified HBsAg was serially diluted (from 2.37ug/ml) and applied along the 4 rows up to column 12. HRP-linked anti-HBs conjugates were applied to each row as follows: goat (lab prepared), rabbit; monoclonal (mouse) and goat (commercial). The goat (commercial) conjugate showed the highest signal

The performance of the goat anti-HBs conjugate acquired commercially was compared with that of commercially acquired ovine HRP-linked anti-HBs conjugate in wells blocked with BSAT. The ovine HRP-linked anti-HBs conjugate / BSAT system gave a far much higher signal with HBsAg positive sample and slightly lower background signal than the goat anti-HBs conjugate / BSAT system, which did not give any notable positive signal (Figure 4.6). This showed that the goat anti-HBs conjugate and BSAT were not

compatible. These results agreed with an observation by Crowther (2001) that there is no single blocking buffer that is ideal for every occasion as each antibody- antigen pair has unique characteristics. The trend of the ovine HRP-linked anti-HBs conjugate / BSAT system was not regular at lower concentrations of HBsAg positive samples possibly due to experimental errors such as irregular mixing of the conjugates.

Since the ovine HRP-HBs conjugate / BSAT system had showed desirable performance (Figure 4.6) and the goat anti-HBs conjugate / BSA system had shown fairly good performance (Figure 4.5), the two systems were compared by running them in parallel assay. The ovine HRP-HBs conjugate / BSAT system gave a far much higher signal with the HBsAg positive sample and slightly lower background signal than the goat anti-HBs conjugate / BSA system (Figure 4.7). The duplicates of the test also displayed very close pattern signifying that the test was reliable.

From these results, a decision was made to pick ovine HRP-HBs conjugate / BSAT system for use in the subsequent stages of the development of the kit.

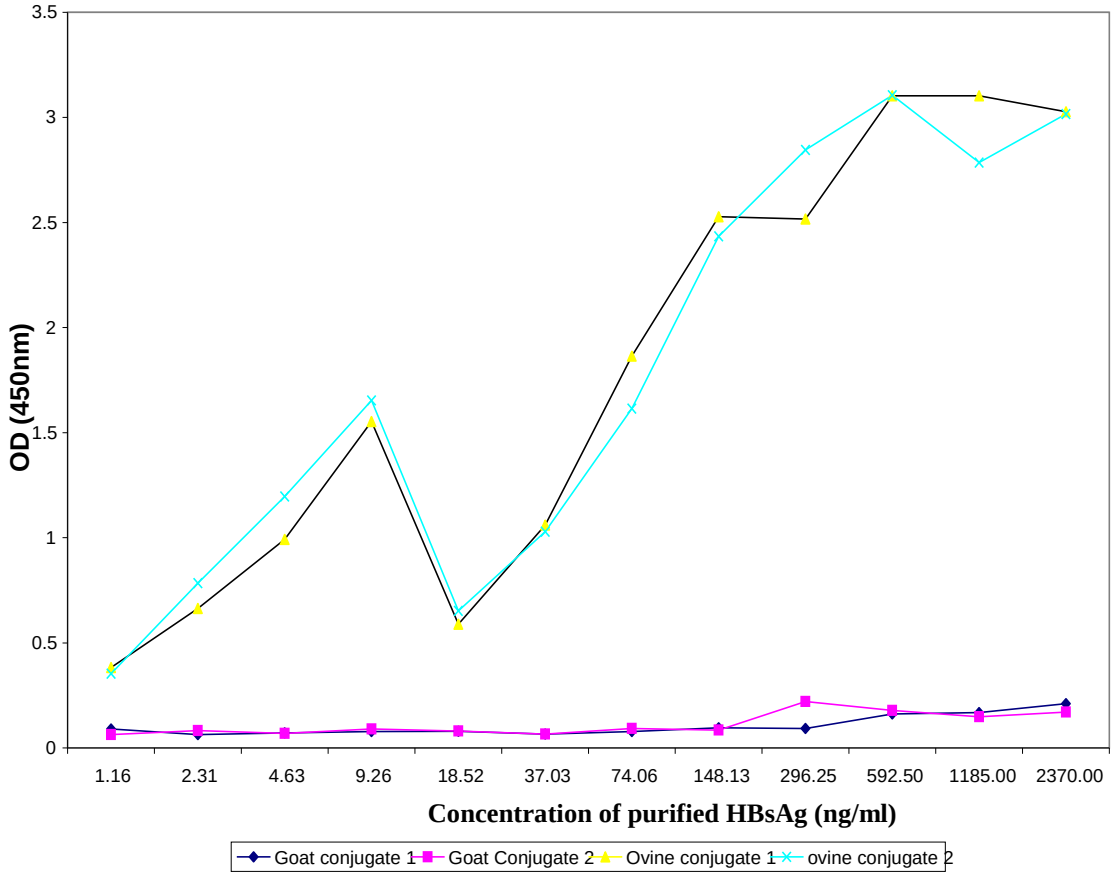


Figure 4.5 Performance of ovine and goat HRP-linked anti-HBs conjugates in BSAT blocked wells.

Four rows of ELISA plate were run with the following parameters: coating with anti-HBs (5µg/ml); blocking with BSA (rows 1&2) and BSAT (rows 3&4); goat HRP-linked anti-HBs conjugate (rows 1&2); ovine HRP-linked anti-HBs conjugate (rows 3&4); and TMB-Urea Peroxidase. Ovine HRP-linked anti-HBs conjugate / BSAT system was far much more sensitive. P-values; ovine conjugate- 0.9032; Goat conjugate - 0.98257

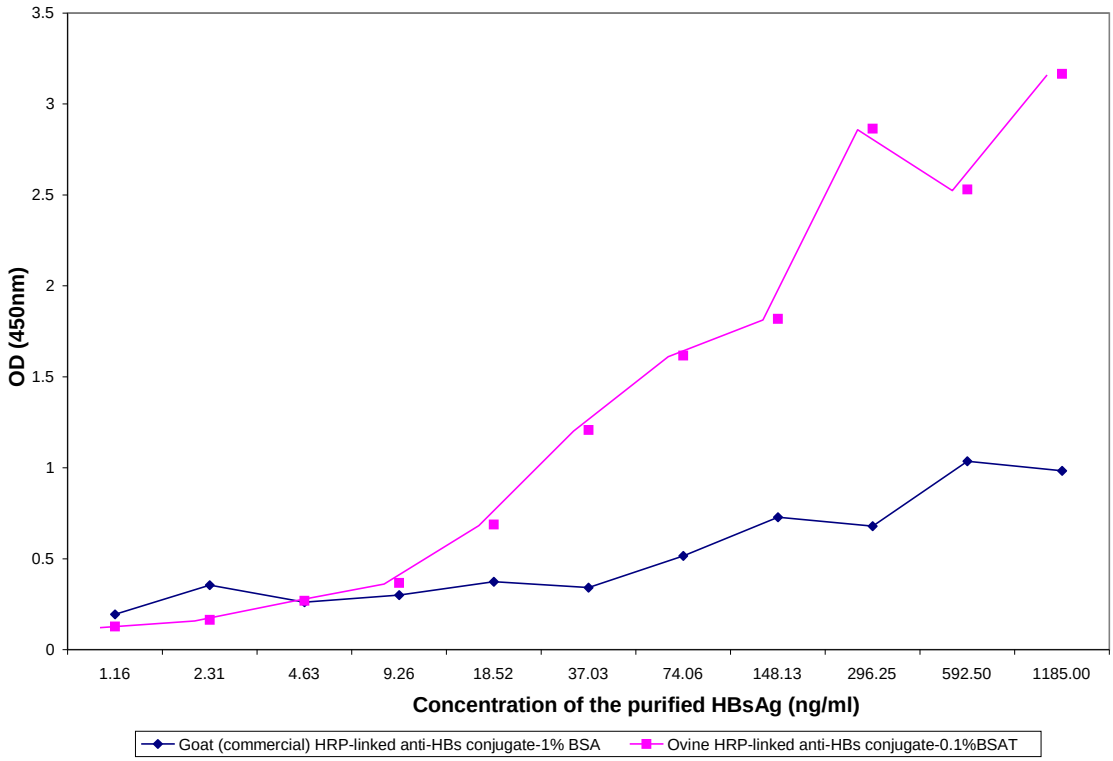


Figure 4.6 Performance of the goat HRP-linked anti-HBs conjugate/ BSA and ovine HRP-linked anti-HBs conjugate/BSAT systems

An ELISA was run with the following parameters: 5µg/ml of coating anti-HBs; goat (commercial) HRP-linked anti-HBs conjugate and BSA system (duplicate); ovine HRP-linked anti-HBs conjugate and BSAT system (duplicate); 2-fold serially diluted purified HBsAg. The ovine HRP-linked anti-HBs conjugate and BSAT system was more sensitive. P-values: Goat conjugate - 0.9463; ovine conjugate - 0.990, $\alpha \leq 0.001$.

4.1.5 Selection of the 3, 3', 5, 5'tetramethylbenzidine Substrate

Two TMP substrate systems were tested: TMB-Urea peroxide and TMB- Hydrogen peroxide system by running the tests in parallel. There were no marked differences in the pattern and the levels of the absorbance in the two TMB substrate systems (Pearson Correlation 0.982, $\alpha \leq 0.01$, 2-tailed). These results are in agreement with the observation by Tai (1992) that the main difference between the TMB-Urea peroxide and TMB-

Hydrogen peroxide system lies in their stability not in their performance in the assay system.

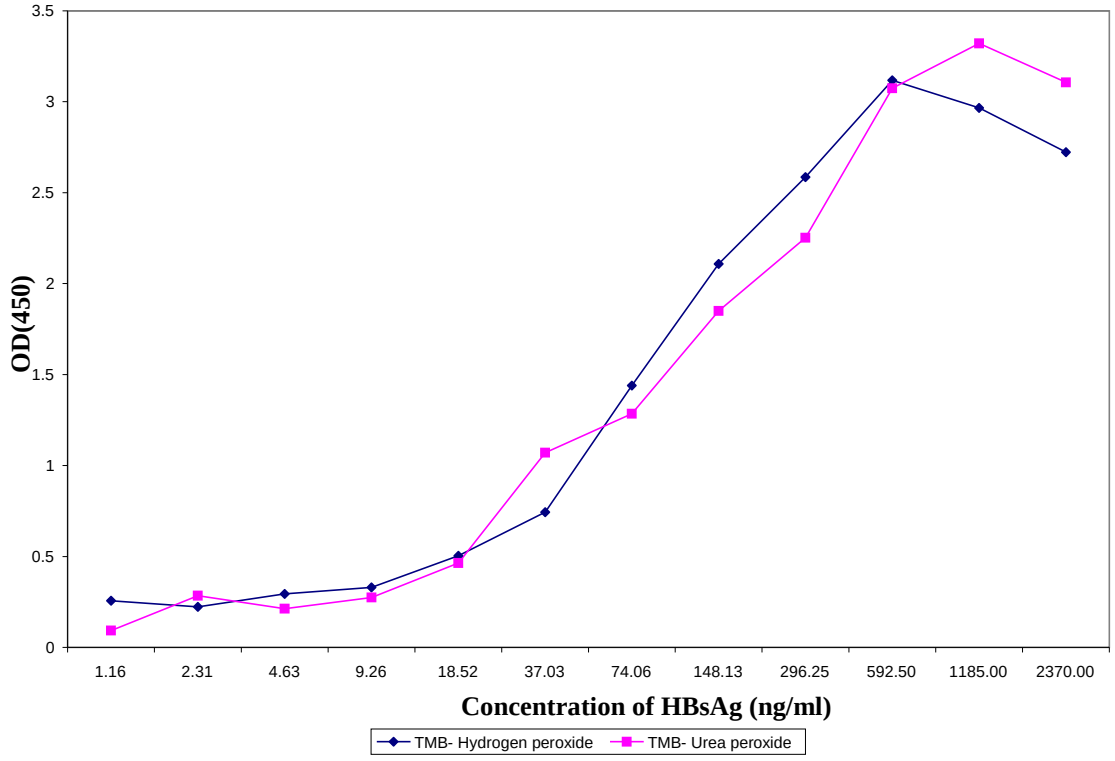


Figure 4.7 Comparison of the performance of the TMB-Urea peroxide and TMB- Hydrogen peroxide ELISA systems

An ELISA was run with the following parameters: anti-HBs coating (5µg/ml); BSAT blocking buffer; Ovine HRP-linked anti-HBs conjugate; TMB-Urea peroxide and TMB-Hydrogen peroxide substrates in duplicate. The mean ODs were calculated for the corresponding rows to produce the graph below. There was no marked difference in pattern of ODs in the two TMB substrate systems. Pearson Correlation 0.982 ($\alpha \leq 0.01$, 2-tailed).

4.1.6 Determination of initial Cut-off value

The cut-off value was calculated using the method described by Greiner *et al.* (1994).

The absorbance values in 11 wells (A1, A2, A3, A4, A5, A6, A7, A8, A9, A10, A11) where pooled negative control plasma was loaded were: 0.085, 0.088, 0.082, 0.086, 0.078, 0.078, 0.079, 0.079, 0.079, 0.122 and 0.099

The mean absorbance value was 0.088.

0.2 was added to the calculated mean, 0.088 (Greiner *et al.,* 1994).

Cut-off = 0.2 + 0.088 **= 0.288**

Samples were regarded as positive when absorbance values were at least 10% (0.316) greater than the cut-off value and negative if at least 10% (0.259) less than the cut-off value and equivocal if within 10% (0.259 – 0.316) of the cut-off value (Greiner *et al.,* 1994).

The absorbance values in 11 wells where pooled positive control plasma was loaded were: 2.862, 2.984, 3.095, 3.196, 3.22, 3.174, 3.013, 2.919, 2.998, 2.895 and 2.931 with the average absorbance of 3.026 far above the determined cut-off value.

4.3 Optimization of immunoreactants concentrations by checkerboard titrations

The appearance of the ELISA plate in which the anti-HBs from Guinea pig had been used as capture antibodies and HRP-linked anti–HBs (ovine) conjugate used as a detection antibody in a checkerboard technique is shown in Plate 4.4. The plate showed a characteristic Checkerboard titration pattern (Crowther, 2001a) where the optical densities decrease systematically down the column, although well no. D12 gave an outlier result with high colour intensity of a false positive sample that might have been caused by contamination with HBsAg positive sample.

The absorbance values of all the wells are shown in Table 4.4 and Figure 4.9. The main drop of ODs occurred from dilution of the capture anti-HBs of 2.5µg/ml given in column 3 (*Table 4.4).* This implies that the use of higher concentrations of capture anti-HBs did not add any value as all the available binding sites had been saturated above this concentration. Table 4.4 and Figure 4-9 also show that the highest OD value before a major drop among the rows occurred in row 4 where the commercial ovine HRP-linked anti-HBs conjugate was diluted 8 times.

The optimized parameters obtained from this experiment were the capture anti-HBs at the concentration of 2.5µg/ml and the ovine HRP-linked anti-HBs conjugate at a dilution of 1: 8.

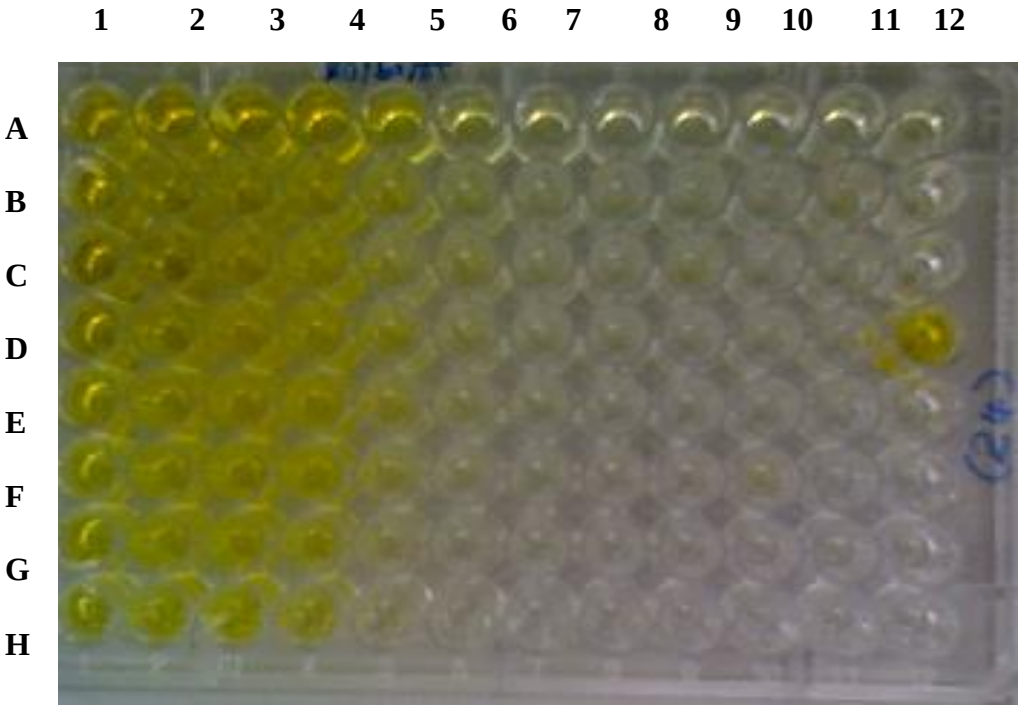


Plate 4.4 The appearance of a developed ELISA plate - checkerboard titration

The capture antibody was serially diluted and applied along the rows while the ovine HRP-linked anti-HBs conjugate was serially diluted and applied along the columns

Table 4.4 The ODs of the wells of the ELISA plate in the checkerboard titration

The capture antibody was serially diluted and applied along the rows while the ovine HRP-linked anti-HBs conjugate was serially diluted and applied along the columns. The ELISA was developed to obtain the OD values shown. The optimum concentration is given by the 4th dilution of ovine HRP-linked anti-HBs conjugate and 3rd dilution of capture antibody (represented by OD of 2.197)

		ELISA plate column numbers *Direction of serial direction of capture antibody* →											
ELISA plate row numbers ***Direction of serial ovine HRP-linked anti-HBs conjugate*** ↓		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
	A	2.559	2.68	**2.848**	2.021	0.598	0.288	0.2	0.126	0.139	0.129	0.117	0.145
	B	2.013	2.194	**2.457**	1.517	0.455	0.273	0.167	0.149	0.144	0.104	0.142	0.134
	C	2.184	2.135	**2.538**	1.182	0.288	0.278	0.16	0.125	0.185	0.105	0.108	0.096
	D	**2.011**	**2.329**	2.197	1.233	**0.399**	**0.21**	**0.125**	**0.114**	**0.106**	**0.108**	**0.097**	**0**
	E	1.293	1.669	**1.812**	0.927	0.332	0.191	0.128	0.094	0.094	0.103	0.088	0.13
	F	0.791	1.205	**1.333**	0.842	0.288	0.139	0.11	0.092	0.096	0.155	0.073	0.082
	G	0.838	1.143	**1.119**	0.542	0.192	0.111	0.091	0.082	0.086	0.075	0.082	0.078
	H	0.659	0.798	**0.825**	0.479	0.178	0.098	0.081	0.07	0.066	0.066	0.067	0.069

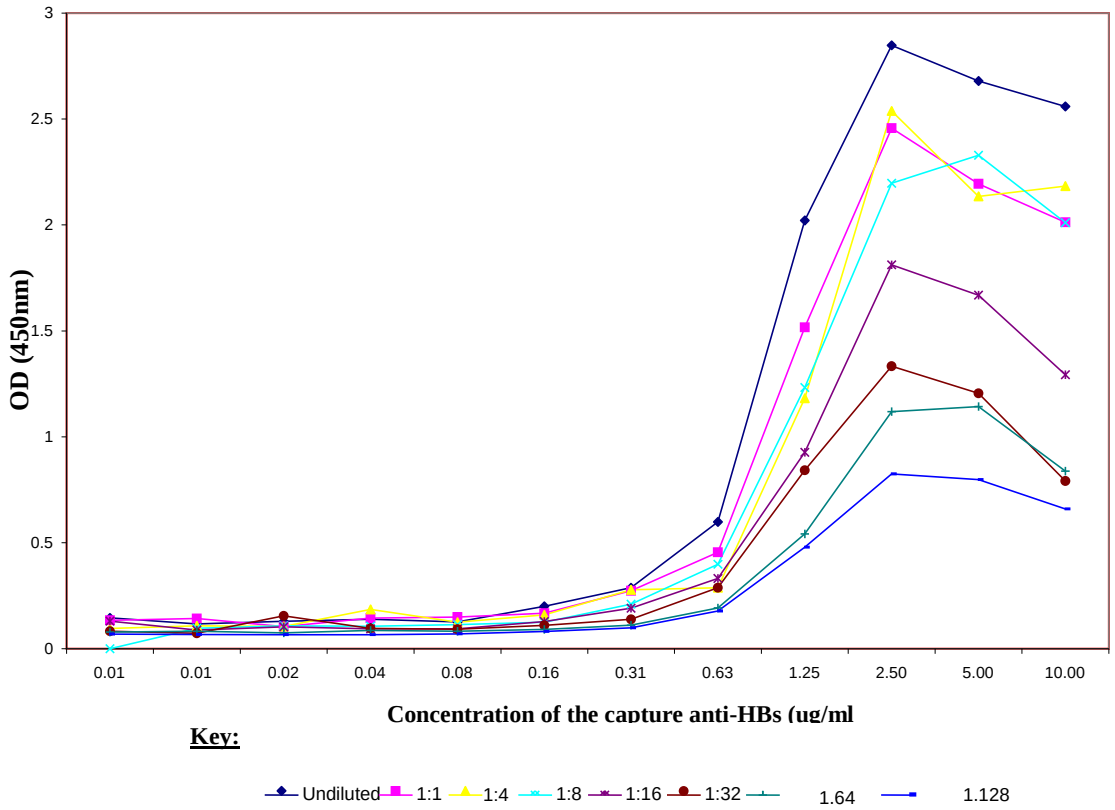


Figure 4.8 The graphical presentation of the checkerboard titration results

The capture antibody was serially diluted and applied along the rows while the ovine HRP-linked anti-HBs conjugate was serially diluted and applied along the columns. The optimum concentration is given by the 4th dilution of ovine HRP-linked anti-HBs conjugate and 3rd dilution of capture antibody

4.4 Analytical sensitivity and specificity

The following were the calculations of the concentration of the working standard of the purified HBsAg following the technique described by Stoscheck (1990):

Mean absorbance value of purified HBsAg = 0.0282 + 0.0284 + 0.0289 = 0.0283

Concentration of HBsAg = 0.0283 / 2.99 mg/ml = 0.00946mg / ml = 9.46µg/ml

Before running the test the sample was diluted 4 times hence the hence the working concentration was 2.37µg/ml. The theoretical analytical sensitivity is up to the 9th dilution

(1/512) as shown in Figure 4.10. Using the working concentration 2.37µg/ml the estimated analytical sensitivity was 2.37/512 µg/ml = 4.62 ng/ml (Figure 4.10)

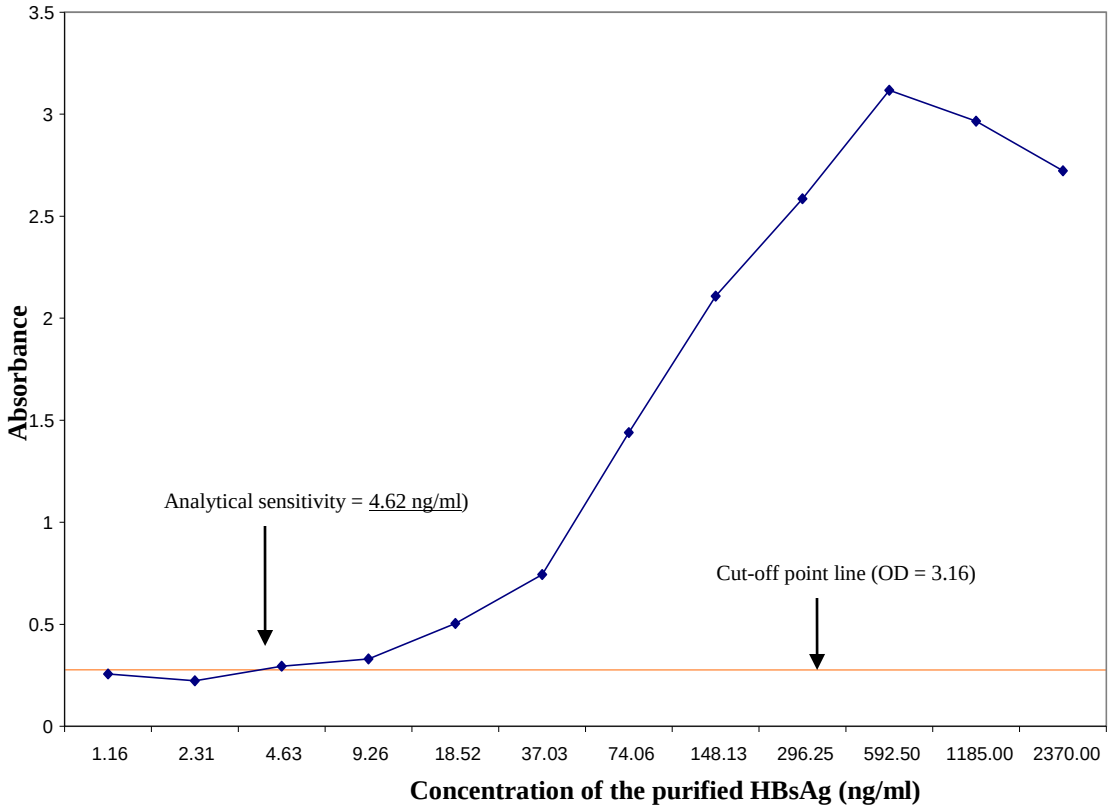


Figure 4.9 A graph to determine analytical sensitivity of the developed ELISA kit

18.92ug/ml of purified HBsAg sample was serially diluted along the row up to the 12th well. The ELISA was run with the established system of BSAT blocking buffer, ovine HRP-linked anti-HBs conjugate and TMB/ peroxide substrate. The analytical sensitivity was found to be 4.62ng/ml.

Figure 4.11 shows the results of comparison of the performance of the developed ELISA kit and the Hepanostika HBsAg Ultra ELISA kit. Although the Hepanostika HBsAg Ultra ELISA kit was slightly more sensitive the gap between them was quite close especially at lower dilutions of the purified HBsAg hence the performance of the developed kit is comparable to that of Hepanostika HBsAg Ultra ELISA kit.

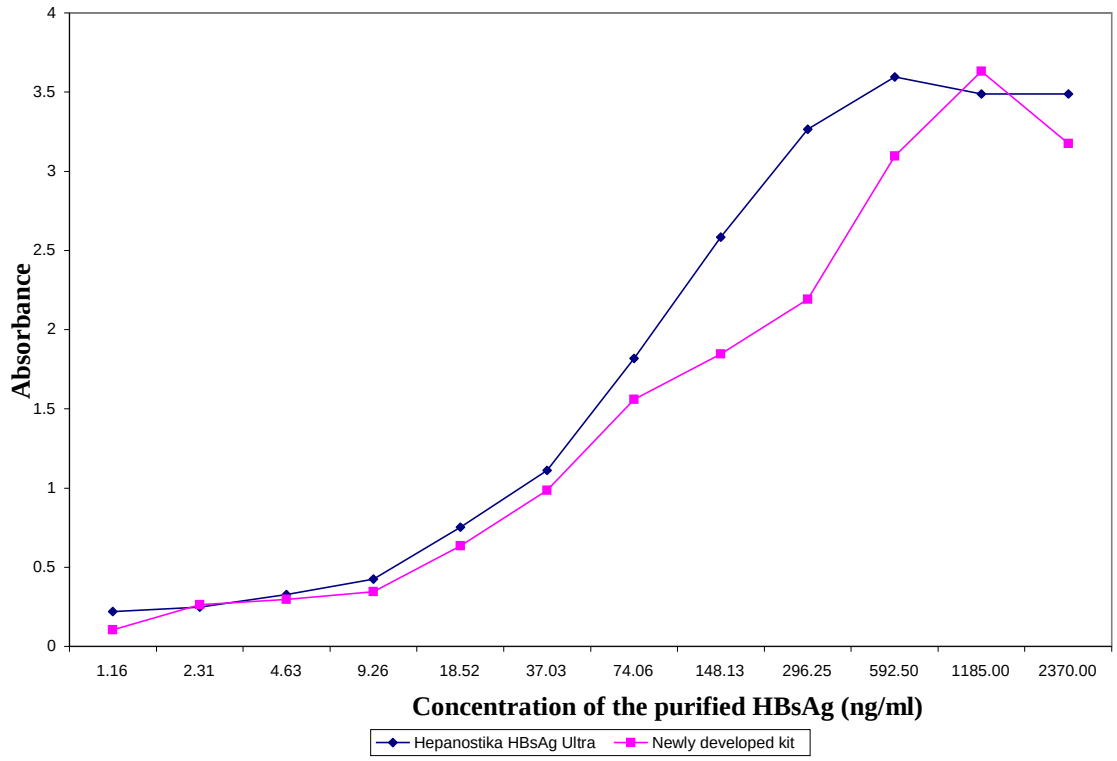


Figure 4.10 Comparison of performance of the developed and the Standard kit

The ELISA of the developed kit was run in parallel with the Standard ELISA kit using a purified HBsAg at the dilution of 1:4 dilution. The general trend of the two curves of the graph was comparable and the curves merge after the 10^{th} dilution.

4.5 Analytical specificity

Analytical specificity was determined by testing Human Immunodeficiency Virus (HIV) and (Hepatitis C Virus (HCV) positive samples in 4 wells of each viral specimen. The absorbance values obtained were 0.273, 0.102, 0.099, 0.089 for HIV positive plasma and 0.076, 0.115, 0.096, 0.082 for HCV positive plasma. They were all below the calculated cut-off point of 2.88 (Figure 2.7)

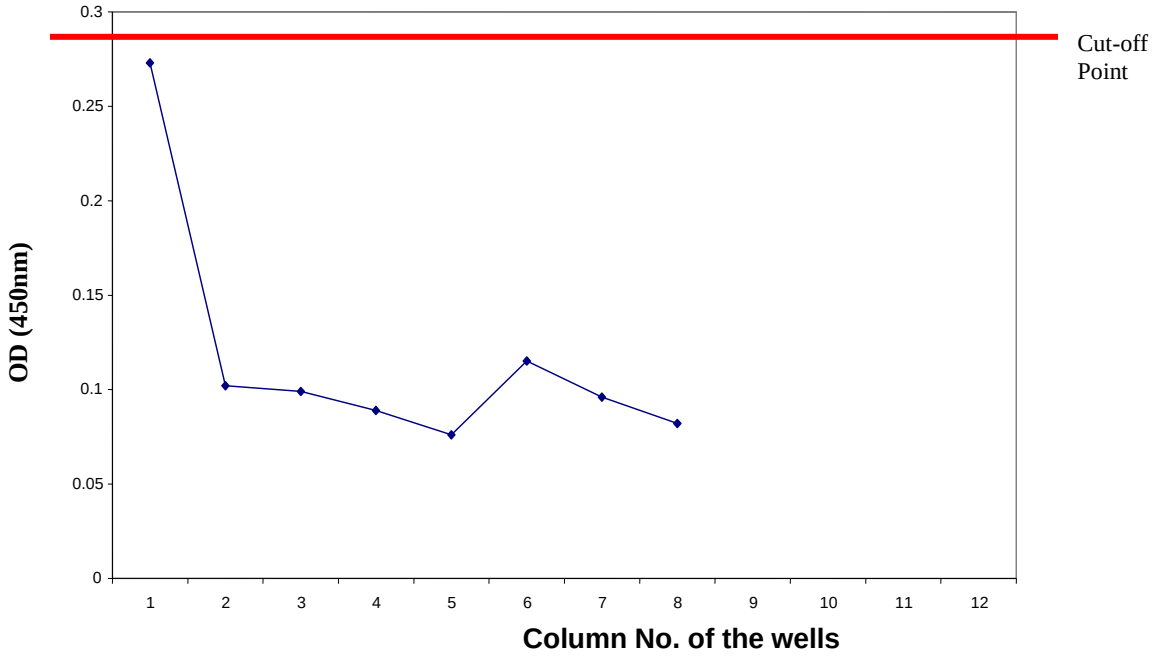


Figure 4.11 The graph of OD of wells where the HIV and HCV positive samples were applied

The ELISA of the developed kit was run against 4 panel sera of HIV and HCV respectively. The OD values for all the samples were below the cut-off point. The high OD value of the first well was possibly due to the "edge effect".

4.6 Diagnostic sensitivity and specificity

A set of 96 samples, 72 plasma (applied in rows A, B, C, D, E and F) and 24 sera (applied in rows G & H) were analyzed using the kit developed in this project and HEPANOSTIKA HBsAg Ultra® for comparative performance (Tables 4.5 and 4.6). The two developed plates for the ELISA Kit and HEPANOSTIKA HBsAg Utra® are shown in Plates 4-5 and 4-6 respectively. Visual examination of the two plates showed marked similarities in colour patterns of the wells.

The kit under development showed that 50 samples were positive and 46 samples were negative. The HEPANOSTIKA HBsAg Utra® showed that 52 samples were positive and 44 were negative. Using HEPANOSTIKA HBsAg Utra® as the Gold standard the kit under development showed the diagnostic sensitivity of 96.1%, diagnostic specificity of 100%, positive predictive value of 100% and negative predictive value of 95.7%.

Table 4.5 The Absorbance values of the ELISA Kit under development

	1	2	3	4	5	6	7	8	9	10	11	12
A	3.086	3.046	3.028	0.088	3.329	3.01	3.366	0.067	0.081	3.065	0.064	2.993
B	2.771	0.318	0.32	0.079	0.1	0.073	0.083	3.045	2.819	2.886	3.081	0.071
C	0.119	0.071	2.026	0.067	0.077	0.065	0.076	0.083	0.068	0.069	3.072	3.169
D	2.841	3.339	3.198	2.737	0.068	0.071	3.174	0.068	0.077	0.072	3.073	0.106
E	3.065	3.241	0.072	0.065	0.071	0.078	1.319	0.068	2.926	3.03	0.077	2.667
F	3.069	3.237	3.166	3.069	3.105	3.087	0.085	0.075	0.077	3.02	0.065	3.263
G	0.124	3.054	0.074	0.07	0.076	0.07	2.676	3.107	3.088	3.236	0.083	3.355
H	0.101	0.082	0.074	0.076	3.243	0.082	0.078	0.369	3.301	3.243	3.192	3.447

Positive – 50
Negative -46

Table 4.6 The Absorbance values of the HEPANOSTIKA HBsAg Ultra

	1	2	3	4	5	6	7	8	9	10	11	12
A	3.342	3.153	3.208	0.243	3.208	3.105	3.041	0.112	0.172	3.342	**0.741***	0.509
B	3.515	1.375	1.527	0.143	0.253	0.111	0.22	3.003	3.003	2.987	3.038	0.177
C	0.151	0.077	3.3	0.072	0.184	0.084	0.219	0.269	0.215	0.234	3.038	2.999
D	3.35	3.128	3.225	3.174	0.127	0.106	3.253	0.248	0.162	0.153	3.084	0.186
E	3.341	3.341	0.282	0.148	0.104	0.138	3.098	0.098	2.989	3.098	0.184	2.929
F	3.489	3.443	3.239	3.489	3.329	3.239	0.176	0.117	0.233	3.329	**0.746***	3.164
G	0.233	3.298	0.14	0.164	0.222	0.205	3.49	3.102	3.365	3.746	0.124	3.403
H	0.239	0.13	0.102	0.096	3.309	0.091	0.235	3.219	3.344	3.309	3.247	3.309

Positive – 52
Negative – 44

* Wells of disagreement between the two tests

Plate 4.5 Pattern of the wells of the developed ELISA kit

ELISA plate was coated with 2.5ug/ml of KEMRI generated anti-HBs. The plate was blocked with BSAT. Samples of well characterized panel sera were applied in each marked well. Ovine HRP-linked anti-HBs conjugate was applied and signal detected using TMB – Hydrogen peroxide substrate.

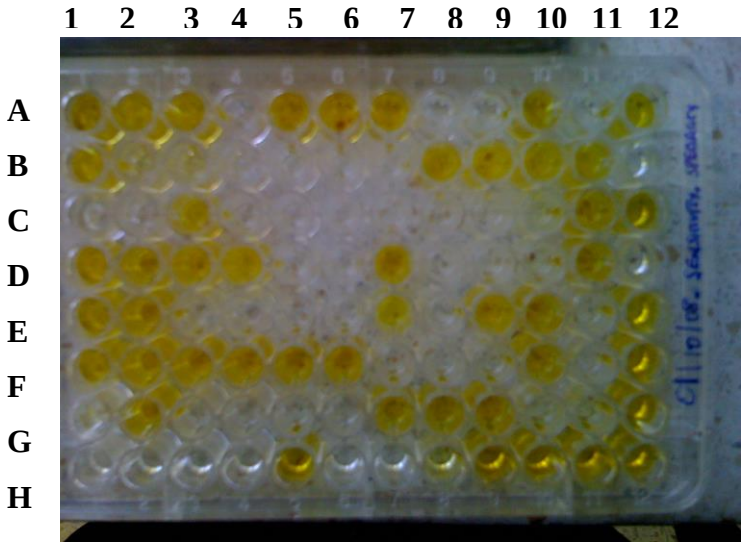
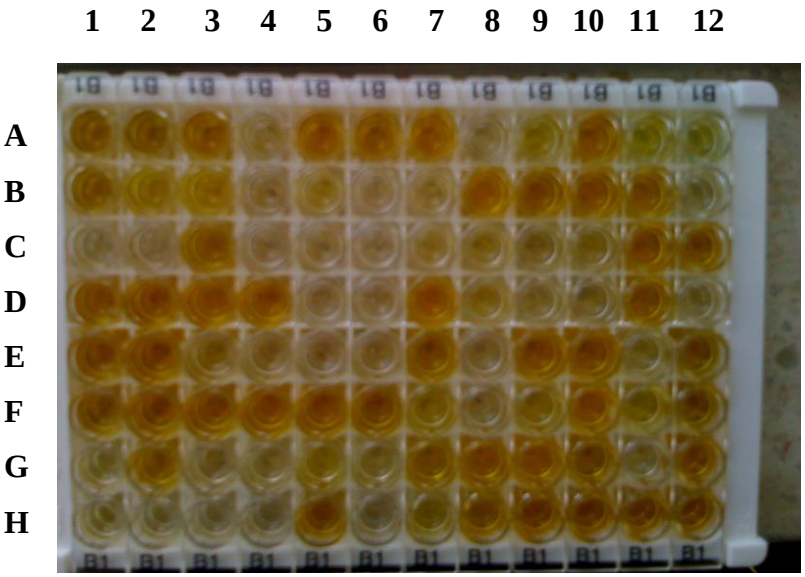


Plate 4.6 Pattern of the wells of the HEPANOSTIKA HBsAg Ultra® ELISA kit

Samples of well characterized panel sera were applied in each marked well and ELISA procedure was carried out



Comparison of the results of carrying the tests using the characterized samples of serum and plasma on both kits (new kit and the standard) showed a Correlation coefficient, r, of 0.933 for sera and 0.929 for plasma (at $\alpha \leq 0.001$) indicating close relationship between the results from the two samples. The p-value was 0.925 for new kit and 0.890 (at $\alpha \leq 0.001$) and hence supporting the theory that there was difference between the use of serum samples and plasma samples.

Table 4.7 2 x 2 contingency table for D-SN, D-SP, PPV and NPV

		Condition (as determined by "Gold standard")		
		True	*False*	
Test outcome	*Positive*	50	0	**50**
	Negative	2	44	**46**
		52	**44**	**96**

Diagnostic Sensitivity % = $\frac{50 \times 100}{52}$ = 96.1 %

Diagnostic Specificity % = $\frac{44 \times 100}{44}$ = 100 %

Positive Predictive Value % = $\frac{50 \times 100}{50}$ = 100%

Negative Predictive Value % = $\frac{44 \times 100}{46}$ = 95.7 %

* Calculations done according to Crowther (2001a)

4.7 Repeatability and reproducibility

These parameters were calculated as shown below:

4.7.1 Intra-run repeatability

The HBsAg sample was applied on the two rows of the ELISA plate and ELISA procedures was carried out as indicated in the *Appendix 3* with and the optimized parameters to obtain the optical densities given in Table 4.8. The coefficients of variance (CV) for each row and for both rows were calculated using SPSS version 12.0.1 for Window. The mean CV was found to be 1.38%

Table 4. 8 Data for intra-plate repeatability

Well no.	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	Mean	Std. Deviation
Run 1 (OD)	1.404	1.453	1.418	1.457	1.413	1.454	1.406	1.413	1.440	1.410	1.405	1.399	1.423	0.0218
Run 2 (OD)	1.471	1.437	1.438	1.425	1.406	1.415	1.410	1.420	1.421	1.423	1.416	1.404	1.424	0.0183

CV for run 1 = 1.54%

CV for run 2 = 1.28%

CV for run 1 & 2 = 1.38%

4.7.2 Inter-run repeatability

Three replicates of each sample were run with five plates on five separate occasions. Coefficients of variation was found to be 5.3%

Table 4.9 Inter-run repeatability

	day 1			day 2			day 3			day 4					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
sample 1	1.406	1.454	1.422	1.461	1.404	1.519	1.394	1.403	1.393	1.411	1.405	1.394	1.312	1.258	1.28
sample 2	1.479	1.431	1.446	1.493	1.439	1.442	1.409	1.428	1.428	1.429	1.433	1.398	1.291	1.31	1.374
sample 3	1.197	1.169	1.178	1.153	1.153	1.086	1.055	1.034	0.996	1.305	1.202	1.255	1.175	1.218	1.158

	Mean	Std Dev	Coefficient of variance %
Sample 1	**1.3944**	**0.06732**	**4.8**
Sample 2	**1.4153**	**0.05475**	**3.9**
Sample 3	**1.1556**	**0.082921**	**7.2**
Coefficient of variance %			**5.3**

4.7.3 Inter-subject variability

Inter-subject variability was determined by giving two analysts the developed kit to carry out similar tests and Kappa statistics used to calculate level of agreement as follows:

Table 4.10 Inter-subject variability

Analyst A (1st analyst)

Analyst B (2nd analyst)	Positive for HBsAg	Negative for HBsAg	Total
Positive for HBsAg	50 (26)	0	50
Negative for HBsAg	0	46 (24)	46
Total	50 (52.1%)	46	96

Cohen's kappa formula (Sim and Wright, 2005) used is:

Kappa = (Observed agreement - Chance agreement)/ (1 - Chance agreement)

= (100% - 50%)/100% - 50%)

= 1

This signifies perfect agreement (Kappa coefficient or Kappa statistic of 1)

4.8 Comparison of performance of the three kits

Tests were ran as explained in *section 4.6* of this study using HEPCELL® PHA and HEPCELL Rapid® instead of the developed kit and HEPANOSTIKA HBsAg Utra® respectively.

Pearson Correlation was calculated using SPSS version 12.0.1 for Window

Table 4.11 Comparison of performance of the three kits

Correlations

		STD Kit	New kit	HEPCELL PHA	HEPCELL RAPID
STD Kit	Pearson Correlation	1	.917(**)	.859(**)	.917(**)
	Sig. (2-tailed)		.000	.000	.000
	N	96	96	96	96
New kit	Pearson Correlation	.917(**)	1	.939(**)	1.000(**)
	Sig. (2-tailed)	.000		.000	.000
	N	96	96	96	96
HEPCELL PHA	Pearson Correlation	.859(**)	.939(**)	1	.939(**)
	Sig. (2-tailed)	.000	.000		.000
	N	96	96	96	96
HEPCELL RAPID	Pearson Correlation	.917(**)	1.000(**)	.939(**)	1
	Sig. (2-tailed)	.000	.000	.000	
	N	96	96	96	96

** Correlation is significant at the 0.01 level (2-tailed).

The developed kit has a perfect Pearson correlation (one) with the HEPCELL Rapid and correlation of 0.917 with the gold standard kit, signifying significant correlation. This correlation is closer than that of the HEPCELL RPHA and gold standard kit.

4.8 Prices of HBsAg detection kits in Kenya and costing of the developed product

The Table 4.12 shows the prices of various HBsAg detection kits in the Kenyan Market. The prices for items number 1, 2, 3 and 4 were sourced from HASS SCIENTIFIC Ltd as on 30/11/2008 while that of items 5 and 6 were sourced from KEMRI. The expected price of the developed HBsAg detection kit is given as item no.7 calculated as shown in *Appendix 6*.

Table 4.12 Prices of HBsAg Detection Kits in Kenya

	Name	**Manufacturer**	**Test type**	**Pack size**	**Price per pack (USD**	**Cost per Test (USD)**
1.	Determine Hepatitis B®	Abbot Diagnostics	Immunochrom-atographic	100	100.000	1.000
2.	Eurotex®	EUromedic	Slide Test	100	62.500	0.625
3.	Hepanostika HBsAg Utra®	Biomerieux	ELISA	192	311.650	1.613
4.	HBS AG	PBS-Orgenics	ELISA	96	80.000	0.838
5.	Hepcell RPHA®	KEMRI	RPHA	200	125.000	0.625
6.	Hepcell Rapid®	KEMRI	Immunochrom-atographic	20	17.500	0.875
7.	**Developed HBsAg detection Kit**	**KEMRI**	**ELISA**	**96**	**50.000**	**0.520**

The maximum price of the ELISA kits considered in the study was USD 1.6125 for Hepanostika Ultra HBsAg kit with the P- value of 0.96 implying that the prices were significantly different (at $\alpha \leq 0.05$).

CHAPTER FIVE

5. DISCUSSION

5.1 Coating of ELISA plates and carrying out the initial Sandwich ELISA Test

5.1.1 Binding of the anti-HBs on the ELISA plate

The HBsAg polyclonal antibodies generated in KEMRI were successfully coated on the ELISA plate and the use of such antibodies to make HBsAg ELISA is widely used mentioned (Wolters *et a*l., 1976; Anderson *et al.*, 1983). The background signal shown by the pooled negative control was generally high and it decreased with the decreasing concentration of the capture anti-HBs. These interfering substances were closely associated with the anti-HBs and could not be washed off. The substances were possibly anti-IgGs such as rheumatic factors (Crowther, 2001b). The blocking process of the void spaces unoccupied by anti-HBs was effective as the background signal in wells with lower concentration of coating anti-HBs was minimal. From the trend of absorbance and after consideration of the initial concentration of anti-HBs used, the provisional working concentration of the capture anti-HBs was adopted as 5μg/ml as the plateau had stabilized at this concentration and use of higher concentrations of anti-HBs would have been a waste.

5.1.2 Performance of various blocking agents

Assessment of skimmed milk, Bovine Serum Albumin (BSA), Normal Rabbit Serum (NRS) and Normal Guinea Pig Serum (NGPS) blocking buffers that carried out using different batches of guinea-pig generated HBs polyclonal antibodies produced by KEMRI from 1994 to 2007 indicated that 5% skimmed milk had the weakest signal on HBsAg

positive sample while the wells blocked with 1% BSA showed the highest signal with similar samples. Generally wells blocked with 0.3% normal rabbit serum (NRS) and 0.3% normal guinea pig serum (NGPS) showed the highest Signal: Noise Ratio (optical density of positive sample over the optical density of the negative sample) of the samples o as the levels of non-specific binding was substantially lower than in wells where BSA was used. This observation concurred with the one documented by Norland (1986) that blockers consisting of whole normal or pre-immune serum sometimes perform better than the BSA or skimmed milk. This is due to the fact that normal serum contains a wide variety of biomolecules of various molecular weights and configurations such that hydrophobic, ionic, and covalent active sites on the surface are blocked more adequately (Norland, 1986). The experiment also demonstrated that the performance of various lots of anti-HBs generated by KEMRI from 1994 to 2007 was almost the same (p-values: BSA – 0.989; skimmed milk – 0.920; NRS – 0.894 and NGS- 0.940)

Further experiments on the blocking buffers in this study established that the mixed solution containing 1% bovine serum albumin and 0.05% Tween 20 (BSAT) buffer resulted in better blocking effects than either bovine serum albumin alone. These results reflected the observation made by Rosenberg (2004) that sometimes combination of the blocking buffers such as the use of BSA and Tween 20 gives a better blocking profile. From the results, it was decided that the trial was to proceed with BSAT buffer as the working blocking buffer.

The important role played by the compatibility of blocking buffers with other components of the ELISA system, especially the conjugate, in determining the success of

the system was clearly evident from the results shown by Figure 4.6 where the ovine HRP-linked anti-HBs conjugate showed drastically higher signals with HBsAg positive samples in comparison with goat HRP-linked anti-HBs conjugate on plates coated with BSAT in which case the blocker appeared to suppress the signal instead of enhancing it. The results of the study of the blocking buffers are in concordance with the observations made by Crowther (2001a) that there is no single blocking buffer that is ideal for every ELISA system as each antibody-antigen pair has unique characteristics hence the necessity to carry out the trial and optimization of appropriate blocking buffer for development of the HBsAg ELISA kit. Generally the lot numbers of anti-HBs produced by KEMRI showed comparatively close Signal: Noise ratio with the use of the same blocking buffer and none of the lot numbers of the anti-HBs showed the highest signal with more than one blocking buffer. This infers that the anti-HBs produced by KEMRI over the period of 13 years had comparatively close performance index. The results of this agreement were used to make the decision of using 1% / 0.05% BSAT as blocking buffer of choice in the study.

5.1.3 Selection of Conjugate

Four HRP-linked anti-HBs conjugates that were tested in the study namely goat anti-HBs; rabbit anti-HBs; monoclonal anti-HBs (all conjugated in-house) and goat anti-HBs conjugate (acquired commercially) showed varied levels of activities. The goat anti-HBs conjugate acquired commercially conjugated showed the highest signal with HBsAg positive sample albeit with a slightly elevated background signal. The three HRP-linked anti-HBs conjugates prepared in-house by conjugation of goat anti-HBs, rabbit anti-HBs

and monoclonal anti-HBs with Lynx Rapid Conjugation Kits (AbD Serotec) generally showed lower signals.

The performance of the goat HRP-linked anti-HBs conjugate acquired commercially when compared with that of commercially acquired ovine HRP-linked anti-HBs conjugate in wells blocked with BSAT showed that the ovine HRP-linked anti-HBs conjugate / BSAT system gave a higher optical densities values with HBsAg positive sample and lower background signal (optical densities with negative samples) than the goat anti-HBs conjugate / BSAT system with the following P-values: Goat conjugate - 0.9463; ovine conjugate - 0.990, $\alpha \leq 0.001$. This showed that the goat anti-HBs conjugate and BSAT were not compatible. Further comparison of the performance of the ovine HRP-linked anti-HBs conjugate/BSAT system with goat HRP-linked anti-HBs conjugate/BSA system showed that former system was better. Based on this the ovine HRP-linked anti-HBs conjugate/BSAT system was approved for subsequent experiments in the project.

5.1.4 Selection of the TMB Substrate

The TMB-Urea peroxide and TMB-Hydrogen peroxide substrate systems were both found to have relatively similar levels of performance in the ovine HRP-linked anti-HBs conjugate/BSAT blocking buffer system with Pearson Correlation of 0.982 ($\alpha \leq 0.01$, 2-tailed). Urea peroxide, which is a combination of urea and hydrogen peroxide in equal amounts, breaks down into urea and hydrogen peroxide and hence providing a more stable source of hydrogen peroxide than hydrogen peroxide solution itself (Tai, 1992).

5.1.5 Checkerboard titrations to optimize concentrations of capture and detection

The study showed a characteristic Checkerboard titration pattern as described by Chart and *et al.* (1994) and Pascho *et al.* (1997), although well no. D12 of Plate 4-4 gave an outlier result with high colour intensity that could be misconstrued to be a positive sample. In all the rows the absorbance values increased from column 1 to column 3 (corresponding to the concentration of 2.5µg/ml of the capture anti-HBs) and then fell rapidly to column 5, after which the curve start flattening fast. The curves were all flat by the 8th column with rows showing lower peaks of their absorbance flattening earlier than those showing higher peaks. Generally, the curves flattened at low absorbance values. The highest OD value before a major drop occurred in row 4 where the commercial ovine HRP-linked anti-HBs conjugate was diluted 8 times. From this checkerboard titration, the optimum concentration of capture anti-HBs was established at 2.5µg/ml while that of ovine HRP-linked anti-HBs conjugate was the dilution of 1: 8 of the stock solution.

5.1.6 Repeatability of the diagnostic test

Initial evidence of repeatability, which is defined as agreement between replicates within and between runs of the assay (Jacobson, 1996; Gao *et al.*, 2004) was calculated. The coefficient of variance (CV) within the assay was found to be 1.46% which signifies high level of agreement between replicates within the assay. The coefficient of variance (CV) between the assays of the same sample was found to be 5.3% far much below the maximum expected value of 20% for raw absorbance values signifying high level of repeatability or reliability of the assay as defined by Reed *et al*. (2002)

5.1.7 Diagnostic sensitivity, diagnostic specificity, analytical sensitivity and specificity, and repeatability

The developed kit was found to have diagnostic sensitivity of 96.1%, diagnostic specificity of 100%, Positive Predictive Value 100% and Negative Predictive Value 95.7% with an established HBsAg ELISA kit (Hepanostika HBsAg Ultra) as a Gold standard. There were no notable differences between the outcomes from plasma and that from serum samples with all the kits used in the study. The apparently low sensitivity of the developed HBsAg ELISA kit is possibly due to the use of few samples in the test. Jacobson (1996) recommends that 300 reference samples from known-infected animals (positive samples), and 1,000 samples from known-uninfected animals (negative samples), should be included to determine D-SN and D-SP, respectively. It is notable also that the Gold standard used in this test is based on the use of monoclonal antibodies raised against native wild-type HBsAg and reactive with HBsAg in which the common 'a'-determinant is modified by site-directed mutagenesis of four of the cysteine moieties. This kit is therefore expected, according to Van Roosmalen *et al.* (2006), to detect more variants of HBsAg, an advantage formally enjoyed by the HBsAg ELISA kits based on the polyclonal capture antibody system (Jongerius *et al.*, 1997).

The performance of the developed kit is comparable to the Hepcell® Rapid Immunochromatographic test kit with Pearson Correlation value of 1 (one) and it is superior to Hepcell® RPHA whose Pearson Correlation value of 0.939. The two kits, Hepcell® Rapid Immunochromatographic test kit and Hepcell® RPHA have been evaluated and approved for use in Kenya hence the developed kit stands a good chance of passing similar evaluation and approval for use in the country.

The end-point dilution analysis, which indicates the dilution of serum in which antibody is no longer detectable, was observed between the 10^{th} and 11^{th} dilution of 1:4 dilution of purified HBsAg using a calculated cut-off of 2.88. Analytical sensitivity was estimated to be 4.62ng/ml using laboratory prepared purified HBsAg standard and spectrophotometric absorbance technique (Stoscheck, 1990). The ELISA of the developed kit when run in parallel with the Hepanostika HBsAg Ultra® kit using the same dilutions and specimen of purified HBsAg showed the relatively comparable performance profile of the two ELISA kits although the Hepanostika HBsAg Ultra® had shown slightly higher signal at concentrations of HBsAg above 74ng/ml. The analytical sensitivity of the Hepanostika HBsAg Ultra® had been determined using the internationally recognized standard panel of sera obtained from Agence Française de Sécurité Sanitaire des Produits de Santé (AFSSaPS) and found to be 0.12ng/ml (Van Roosmalen *et al.,* 2006). By comparing the performance of the two kits in Figure 4.11, an assumption could be made that the analytical sensitivity of the developed kit is the approximately 0.246ng/ml by the standard of Agence Française de Sécurité Sanitaire des Produits de Santé (AFSSaPS AFSSaPS. The higher values of 4.61ng /ml could be due to the fact that the final value of the cut-off point had not been determined by using more samples. It is also known that spectrophotometric absorbance technique of determining the concentration of proteins is not an accurate technique and it is mostly used for estimation purpose (Stoscheck, 1990). Analytical specificity tests determined by using HIV and HCV positive samples as per the procedure described by Jacobson (2002) showed no sign of nonspecific reactions in respect to these samples.

5.1.9. Pricing of the developed HBsAg detection ELISA kit

The pricing steps of the new HBsAg ELISA are presented in *Appendix 6*. The grand total cost of production of the kit with 12 x 8 ELISA plates will be $ 50 ($ 0.52 per test) including a profit of 25% and provision of a contingency cost of 10%. The commercial price of the most widely HBsAg detection ELISA kit in Kenya, Hepanostika HBsAg Ultra®, stands at $311.65 for a kit of two 12 x 8 ELISA plates ($1.62 per test) with the P-value of 0.96 implying that its price is significantly different (at $\alpha \leq 0.05$). The cost price of the developed HBsAg detection ELISA kit is only 32% of the cost price of the Hepanostika HBsAg Ultra®. The cheapest ELISA kit available in Kenya is HBS AG which retails at $ 80 per kit of 96 tests ($ 0.84 per test), which is 60.3% more expensive than the expected price of the developed ELISA kit for detection HBsAg with the P-value of 0.46812 implying that its price is not significantly different (at $\alpha \leq 0.05$).

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

An ELISA kit for detection of HBsAg in plasma and serum using polyclonal anti-HBs produced in Kenya was successfully developed with a diagnostic sensitivity of 96.1% and specificity of 100% against a current standard ELISA kit, Hepanostika HBsAg Ultra® (Biomerieux). The performance of the developed kit is comparable to the Hepcell® Rapid Immunochromatographic (KEMRI) test kit with Pearson Correlation value of 1 (one) and it is superior to Hepcell® RPHA (KEMRI) whose Pearson Correlation value of 0.939. The three diagnostic kits;

- Hepcell® Rapid Immunochromatographic test kit
- Hepanostika HBsAg Ultra®
- Hepcell® RPHA)

have been evaluated and approved for use in Kenya hence the developed kit stands a good chance of passing similar evaluation and approval for use in the country.

The cost price of the developed HBsAg detection ELISA kit is 32% of the cost price of the most widely used HBsAg detection ELISA kit in Kenya, Hepanostika HBsAg Ultra®.

From this study, it was evident that the polyclonal antibodies produced by KEMRI bound successfully to the ELISA and that there were minimal variations within and between different lots.

The developed kit did not show any analytical non-specificity with HIV and HCV positive plasma samples.

Previously, there has been no documented evidence of development of an ELISA test kit in Kenya using locally produced primary reagents to a level that can be exploited commercially hence this study has pioneered such development.

The experience gained in this project can also be used in the development and validation of other diagnostic kits that are based or can be based on similar or closely related technology platform to the one used in this study.

6.2 Limitations of the study

- The goat anti-HBs conjugate acquired commercially showed the trend of the graph that was less regular as compared to the others, possibly due to the experimental errors.
- Lack of standard panel of sera from Agence Française de Sécurité Sanitaire des Produits de Santé (AFSSaPS) that would have been used to measure absolute analytical sensitivity of the test.
- Lack of financial resources to acquires reagents to be used to scale up samples sizes of HBsAg positive samples to 300 and HBsAg negative samples to 1000 as recommended by Jacobson (2002) in order to obtain absolute sensitivity of a diagnostic kit.

6.3 Recommendations and Future Prospects

The ELISA kit developed in this study will be tested further in the field for a period of one year before it is applied for registration at the National Public Health Laboratory Services, Nairobi

There is need of validating the developed kit against a number of mutagenic cysteine variants and subtypes on the "a" determinants of HBsAg. A study carried out by Roosmalen *et al* (2006) found that some ELISA kits could fail to detect HBsAg whose common "a" determinants had undergone mutations that affected the 8 cysteine molecules that are believed to form inter- and intra-chain disulfide bridges that divide the "a" determinant into distinct immunological regions. The suggested study will attempt to determine the extent that the developed kit can go to detect these variants.

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APPENDICES

Appendix 1: Materials necessary for use in development HBsAg detection the Kit

	REAGENT	FORMULATION
01	**Coating Buffer**	a) Sodium carbonate ---------- 5.3g Dissolved in distilled water ------ 900ml b) Sodium bicarbonate in solution (a) – 4.2g c) Sodium azide in solution (b) ------ 1g pH ---------- 9.6 d) Distilled water up to ---------- 1000ml
02	**Washing Buffer (20X concentrated)**	a) Tween 20 ---------- 5 ml b) 20X conc. PBS with azide --------- 500 ml
03	**Blocking Buffer**	a) Bovine serum albumin ---------- 1g b) Sodium azide ---------- 0.5g c) PBS up to ---------- 1 litre.
04	**PBS (20x concentrated)**	a) Disodium hydrogen phosphate (hydrated) ---- 341.6g or Sodium hydrogen phosphate (Unhydrous)----135.4g b) Pottasium dihydrogen phosphate ----------51.8g c) Sodium chloride ---------- 98.2g d) Sodium azide ---------- 0.4g. e) Distilled water up to ---------- 1 litre
05	**Sample Diluent**	a) Bovine serum albumin ---------- 1g b) Tween 20 ---------- 0.5ml c) PBS upto ---------- 1000ml
08	**Stop Solution (0.5M Sulphuric acid)**	a) Sulphuric acid (98% pure) ---------- 56 ml b) Distilled water up to ---------- 1 litre This makes 1M sulphuric acid

Appendix 1 continued

09	**Positive Control**	a) Sample ----- Purified human HBsAg b) Required concentration ------- 0.7µg/ml c) Diluent --------- Normal human serum. d) Required volume ---------- 100ml. (target: to give OD value that is about 25% above the cut-off)
10	**Negative Control**	a)Normal Human Serum b)Required volume ---------- 10ml
11	**Conjugate**	HRP conjugated anti-HBsAg antibodies a) Conjugate ---------- 10µl b) PBS ---------- 9,990µl
12	**Substrate**	-TMB Substrate The substrate will be used as it is
13	**Panel sera**	Characterized HBsAg panel samples from production department

Appendix 2: Plate Coating and Blocking

1. Determine the concentration of guinea pig hepatitis B antibody (0.2 - 0.8 μg/ml).
2. Put 100 μl of guinea pig anti-HBs into each well of an ELISA plate.
3. Cover the plate and incubate at room temperature overnight.
4. Wash the plate 4 times with wash buffer.
5. Add 300 μl of blocking buffer into each well.
6. Cover the plate and incubate at room temperature for 3 hours.

   The ELISA plate is now ready for use

7. Keep the plate covered and still with the blocking buffer at 4ºC till use.

Appendix 3: Direct Sandwich ELISA Test

1. Bring reagents, controls and samples to room temperature.
2. Wash the plate 4 times with wash buffer.
3. Add 100µl of each test sample and control into appropriate wells and mix by tapping gently for 1 minute. * *Sample pre-dilution in polypropylene tubes*.
4. Incubate at room temperature for 2 hours.
5. Wash the plate 4 times with wash buffer.
6. Add 100µl of conjugate into each well.
7. Incubate at room temperature for 2 hours
8. Wash the plate 4 times with wash buffer.
9. Add 200µl of substrate into each well.
10. Incubate at room temperature for 30minutes away from direct light.
11. Stop the reaction by adding 100µl of stop solution.
12. Read results using a spectrophotometer at 450nm within 10 minutes.
13. Record the results of each sample and control.

Appendix 4: Preparation of a Standard Curve

1. Determine the concentration of a purified HBsAg using a spectrophotometer.
2. Dilute serially the HBsAg to 1, 2, 4, 8, 16, 32, 64, 128, 256, 512 & 1024µg/ml.
3. Carry out an ELISA test in duplicate using the dilutions as the samples.
4. Plot a curve of HBsAg concentration for each dilution on the x-axis against its OD_{450nm} reading on the y-axis.
5. Use the graph to obtain HBsAg concentrations for different samples from OD_{450nm} readings after carrying out an ELISA test.

Appendix 5: Preparation of Capture Antibody

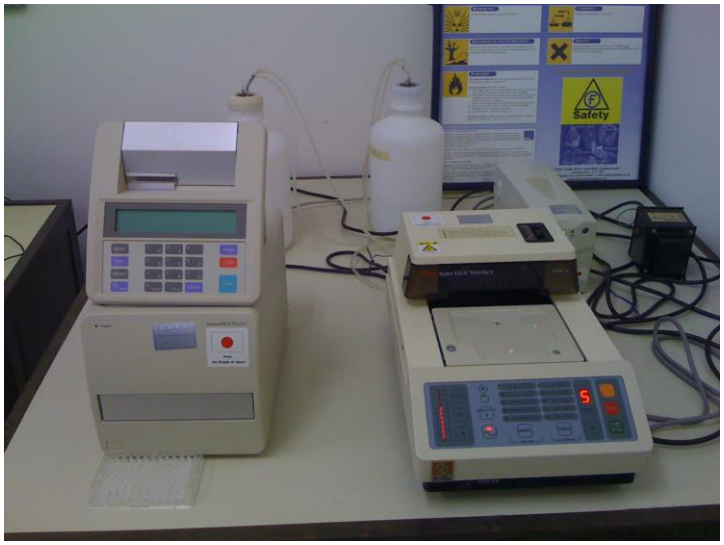
1. Determine the concentration of guinea pig Anti-HBs (polyclonal) using a spectrophotometer.
2. Prepare a required Antibody concentration
3. Prepare a required Antibody concentration in a polypropylene tube using coating buffer as diluent.
4. Keep at 4^{o}C till use.
5. Use 100µl per well when coating the plates for ELISA test

Appendix 6: Costing of the new HBV kit

	Cost item	**Unit cost (USD Per Plate)**
	A. Variable costs	
1.	ELISA plate – 12 x 8 (Sumilon)	1.50
2.	Coating Buffer Ingredients	0.25
3.	Capture Anti-HBs	5.00
4.	Plate covers (3)	0.375
5.	Incubator Power Consumption	0.625
6.	Ovine HRP-linked anti-HBs conjugates	7.50
7.	TMB chromogen	0.625
8.	Urea peroxide	0.375
9.	Sulphuric Acid	0.125
10.	BSAT	0.250
11.	Sodium Azid	0.0625
12.	Labour	13.75
13.	Coding	0.125
14.	Labels for reagents	0.125
15.	Product insert	0.05
16.	Unit Carton / Box	0.375
17.	Negative control	0.375
18.	Positive Control	0.50
19.	Desiccants	0.0625
20.	Washing Buffer	0.250
21.	Sachet	0.125
	Total Variable Costs	**32.425**
	B. Fixed Costs	
	Total Overhead and Depreciation	5.00
	C. Contingency (about 10%)	2.5
	Subtotal	**39.925**
	D. Profit (25%)	**10.00**
	GRAND TOTAL	**40.00**

Appendix 7 : The Laboratory and Equipment used in the study

The Quality Control Laboratory at KEMRI Production Department



ELISA washer and reader used in the study