IMMUNE RESPONSE OF GHANAIAN CHILDREN TO THE HEPATITIS B ANTIGEN IN THE PENTAVALENT (DPT-HepB-Hib) VACCINE IN ACCRA

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DECLARATION

Declaration is hereby made with respect to this thesis that, the work is original and was produced by research carried out at Clinical Virology Laboratory, University of Ghana Medical School, solely by the author under the supervision of Dr Kwamena William Coleman Sagoe and Professor Julius Abraham Addo Mingle. References were made to other authors' work and such authors have been duly acknowledged in the reference section of the thesis. This thesis has not been submitted to any institution in part or whole for the purpose of award of any degree or certificate.

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DEDICATION

Your will oh! Lord be done in my life. Also dedicated to the memory of my late mother Juliana Amoak Apiung

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LIST OF ABBREVIATIATION

ADAMSEL	Auditable Data Analysis and Management System for ELISA
ADHS	Arizona Department of Health Service
ANAE	Anaemia
Anti-HBs	Antibody to Hepatitis B surface Antigen
CDC	Centre for Disease Control and Prevention
CI	Confidence interval (95%)
CTL	Cytotoxic T Lymphocytes
DNA	Deoxyribose Nucleic Acid
DPT-HepB-Hib	Diphtheria Pertussis Tetanus-Haemophilus influenzae b– hepatitis B
ELISA	Enzyme Linked Immunosorbent Assay
EPI	Expanded Program on Immunisation
GAVI	Global Alliance for Vaccine Immunization
GHS	Ghana Health Service
GIT	Gastro intestinal tract
HBcAg	Hepatitis B core Antigen
Ant-HBc	Hepatitis B core Antibody
Anti-HBc IgG	Hepatitis B core Antibody Immune globulin G
Anti-HBe	Hepatitis B envelop Antibody
HBeAg	Hepatitis B envelop Antigen
HBIG	Hepatitis B immune Globulin
HBsAg	Hepatitis B surface Antigen
HBV	Hepatitis B virus

HCC	Hepatocellular Carcinoma
hHBV	Human Hepatitis B virus
HIV	Human immunodeficiency virus
IARC	International Agency for Research on Cancer
KBTH	Korle-Bu Teaching Hospital
MHC	Major Histocompatibility complex
MMWR	Morbidity and Mortality Weekly Report
MOH	Ministry of Health
NC	Negative Control
NK	Natural Killer cells
OD	Optical Density
OTI	Otitis media
PC	Positive Control
PML	Princess Marie Louise Hospital
RT	Room Temperature
RTI	Respiratory tract infection
SRU	Sample ratio units
UTI	Urinary tract infection
VAS	Vascular (diseases affecting part of or whole vascular system)
WHO	World Health Organisation
WWB	Working Wash Buffer
LHB	Large Hepatitis B surface protein
MHB	Medium Hepatitis B surface protein
SHB	Small Hepatitis B surface protein

ABSTRACT

Background: There is no data on the hepatitis B surface antibody responses (anti-HBs) in children after the introduction of the pentavalent (DPT-HepB-Hib) vaccine in Ghana.

Method: Sera from 424 children aged 5 months to 32 months were tested for anti-HBs using an ELISA, (Antisurase B-96, General Biological Corp., Taiwan). A cross-section of those positive for anti-HBs were tested for hepatitis B core antibody (HBcAb) using Foresight® ELISA (Acon laboratories Inc., USA), and those negative for anti-HBs were tested for hepatitis B surface antigen (HBsAg) using Surase B-96 ELISA (General Biological Corp., Taiwan) and Wondfo® rapid screening test (Guangzhou Wondfo Biotech Ltd., China). The primary outcomes of the study were the ability of children to develop a minimum of 10 and 100mIU /ml of anti-HBs. A questionnaire was used to obtain data to determine the correlates for anti-HBs responses.

Results: Of the 424 children, 358 (84.4%) developed anti-HBs while 340 (80.2%) and 205 (48.3%) developed ≥10 and ≥100 mIU/ml anti-HBs titres respectively. A total of 308 (87.0%) of the 354 out of 358 who developed some level of anti-HBs were non-reactive to the test for anti-HBc. Of the 66 who were anti-HBs negative, 3 (4.6%) were HBsAg positive. The use of vaccines from different manufacturers for one individual and the ages of the children, had significant effect on anti-HBs titre ($p < 0.050$). The most significant differences in correlates were seen when a minimum anti-HBs protection of ≥100 mIU/ml was assumed.

Conclusion: There is a need to consider giving a booster hepatitis B vaccine at 9 months and to adhere to protocols for hepatitis B virus prophylaxis in exposed neonates.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Hepatitis B is a viral disease caused by Hepatitis B Virus (HBV), which is a prototype member of the hepatotropic DNA viruses, the Hepadnaviridae. The Hepadnaviridae family is constituted by two main genera namely the *Orthohepadnavirus* that infect mammals e.g. the human Hepatitis B Virus (hHBV) and *Avihepadnavirus* that infect the birds e.g. the Duck Hepatitis B Virus (dHBV) (Glebe and Urban, 2007). Human hepatitis B viral infection is of major public health concern because of the disease burden in some parts of the world particularly, Africa and Asia causing about 500 000 to 1.2 million deaths annually (Zekri et al., 2007). hHBV affects mainly humans with some strains of the *Orthohepadnavirus* family of viruses infecting few mammals including close relatives of man i.e. other primates like Chacma baboons, chimpanzees, gorillas, and tree shrews are also susceptible to infection under experimental conditions (Lyons et al., 2012, Hu et al., 2000, Cao et al., 2003). This blood borne virus with predilection for the liver cells is transmitted sexually, percutaneous and by mucosal exposure to infected body fluids. Infection with HBV causes a cellular immune response leading to the destruction of the liver cells. Progressive degradation of the liver cells results in acute, fulminant, or chronic carrier status with a possible end stage of liver cirrhosis and hepatocellular carcinoma (HCC). Hepatitis B disease severities in infected people usually differ from one individual to the other and may also depend on the genotype of the infecting virus (Bertoletti and Gehring, 2006). The immune systems of about 80% -90%

immunocompetent adult individuals who get infected are able to clear the virus from the blood within six months and ensure effective recovery with or without evidence of acute liver inflammation (Kayser et al., 2005, Bertoletti and Gehring, 2006). Other people who get infected with the virus are not able to clear the virus from the blood and liver and therefore become chronically infected. Most of these chronically infected patients are mainly asymptomatic without life threatening disease making up a large pool of the people who pose a great threat to other susceptible members of the general public in terms of transmission. About 10% to 30% of these asymptomatic carriers normally develop cirrhosis and subsequently HCC (Alberti et al., 1999). Prognosis and pathogenesis of the disease which is largely determined by host and viral factors are difficult to explain. This is because there are few animal models for studies of the virus; that is, the virus has a very narrow alternate host range (Bertoletti and Gehring, 2006).

However, many children under the age of five years who get infected stand the high risk of developing chronic liver diseases, progressing to liver cirrhosis and hepatocellular carcinoma culminating in death, and this usually is after a long period of about 30 to 50 years (*http://www.who.int/csr/disease/hepatitis/whocdscsrlyo 20022/en/index3.html* , IARC MONOGRAPH-100B). In a review of articles on chronic hepatitis B viral infection, 25%-30% of children under six years whose mothers were HBeAg positive, 1%-12% of older children and less than 5% of teenagers and adults become chronic carriers when they are infected (Hyams, 1995); hence, it is important to prevent early childhood infection. Immunization with HBV vaccine is the most effective means of preventing HBV infection and its consequences. The hepatitis B vaccine used to prevent

this disease comes in two forms - monovalent formulation that protect the individual against only hepatitis B and the combined formulation that protects against hepatitis B and other diseases.

The combined vaccine in addition to the HBsAg, have other antigens such as *Diphtheria*, *Pertussis*, *Tetanus*, and *Heamophilus influenzae* type b. The combination could have 2, 4 or 5 antigens appearing as Hib+HepB, DPT-Hep B or DPT-Hep B+Hib. The pentavalent vaccine is used in Ghana which consists of DPT-HepB+Hib antigens. The vaccine is administered in accordance with the timing of the administration of the DPT vaccine that was administered prior to the addition of hepatitis B and *Heamophilus influenza*e type b antigens. The dose administered at birth must be the monovalent formulation whiles both the monovalent and combine formulations can be given later after birth. Providing infants of HBsAg positive mothers with immunoprophylaxis (Hepatitis B Immune Globulin (HBIG) and Hepatitis B vaccine) at birth effectively prevent HBV transmission that could occur during the perinatal period (Stevens et al., 1987, Stevens et al., 1985). Integrating HBV vaccine into childhood vaccination schedules in endemic populations has had great impact in interrupting transmission of the virus in children in Ivory Coast, Senegal, Taiwan, and The Gambia (Magoni et al., 2009, Vildosola et al., 2000, Hsu et al., 1988, Van der Sande et al., 2007).

A recommendation of the World Global Advisory Group of the Expanded Program on Immunisation in 1991 which was endorsed by the World Health Organisation (WHO) assembly in 1992, called for integration of hepatitis B vaccine into countries with high HBsAg prevalence by 1995 and to the rest of the world by 1997 (Zuckerman, 2006,

WHO-EPI, 1996). This led to the integration of HBV vaccine into the Expanded Program on Immunization (EPI) Ghana in 2002 in an attempt to stop transmission of the HBV in the country.

1.2 Epidemiology

Hepatitis B is a common infectious viral disease, about a hundred times more infectious than Human Immune Deficiency Virus (HIV). It is estimated that about one-third (two billion) of the world population has evidence of present or past HBV infection (Dienstag, 2008) of which about 400 million are chronic carriers (McMahon, 2005), and more than one million die annually as a consequence of HBV related liver disease such as cirrhosis, HCC and fulminant hepatitis (Mahoney, 1999). Asia has the largest population of patients with chronic HBV infection accounting for 75% of the world's chronic carriers, while Africa has about 50 million chronic HBV carriers (Blankson et al., 2005). Chronic HBV infection contributes about 50% -90% to HCC which is a common cancer the world over especially in highly HBV endemic areas; hence, it serves as the main contributor to HCC development in chronically infected patients (Chen et al., 1997). The disease burden of hepatitis B in Ghana is usually determined by prevalence studies of chronic carriers.

HBV has been classified into eight main genotypes, designated A to H, and these genotypes are defined by an inter-group sequence divergence of genome sequence of more than 8% (You et al., 2008). Of the eight known HBV genotypes which have distinct geographical distributions, genotype A and E prevail in Africa. While genotype A can be found in sub Saharan Africa and elsewhere in the world, genotype E is mainly found in

Africa (Kramvis et al., 2005). Studies in Ghana has shown varying percentages of the prevalence of hepatitis B genotype E ranging from 87%, to 100% of mostly chronically infected persons (Zahn et al., 2008, Geretti et al., 2010, Candotti et al., 2006, Candotti et al., 2007). However, 10% and 3% of individuals in one of the studies expressed genotype A and genotype D, respectively (Candotti et al., 2006). The differences in clinical appearance, response to treatment and long-term prognosis of HBV infection is dependent on the genotype the patient is infected with explaining the geographic differences in the natural course of chronic HBV infection (Kramvis et al., 2005).

1.3 The virus and the vaccine

The virus was the first hepatitis virus related to humans to be discovered accidentally in1963 by a researcher Baruch Blumberg, looking for inherited serum polymorphic proteins in human blood that indicated susceptibility of these individuals to some diseases like leukaemia, Down syndrome and other cancers of humans (Senior et al., 2011). A protein identified from the blood of an Australian Aborigine which reacted with serum of a New York haemophilic patient was thought to be responsible for most of the post transfusion sickness and subsequently was dabbed the Australian Antigen. Electron microscope was used by Dane and colleagues to discover the virion (42nm infectious particle) that carried the Australian antigen in the blood of patients who were suffering from this Australian antigen and consequently the infective virion was named after him as the “Dane particle.

The disease burden in some endemic parts of the world and the high morbidity and mortality has necessitated vaccination to protect people from the disease. Many countries have embarked on universal infant and child vaccination to stop the development and transmission of the disease. A plasma derived hepatitis B vaccine was developed in the early 1980s and by mid 1980s the recombinant vaccine was produced. There are now many hepatitis B vaccines from different producers available in the market for the prevention of the disease e.g. recombinant hepatitis B vaccine, five-in-one vaccine (pentavalent), Tritanrix-HepB-Hiberix, Engerix-B.

1.4 Prevalence

The WHO classifies areas of the world where the prevalence of HBsAg is >8% as highly endemic, areas with prevalence of HBsAg from 2%-7% and <2% as intermediate and low endemic areas respectively. Figure 1-1 below indicates the parts of the world with their corresponding HBsAg prevalence.

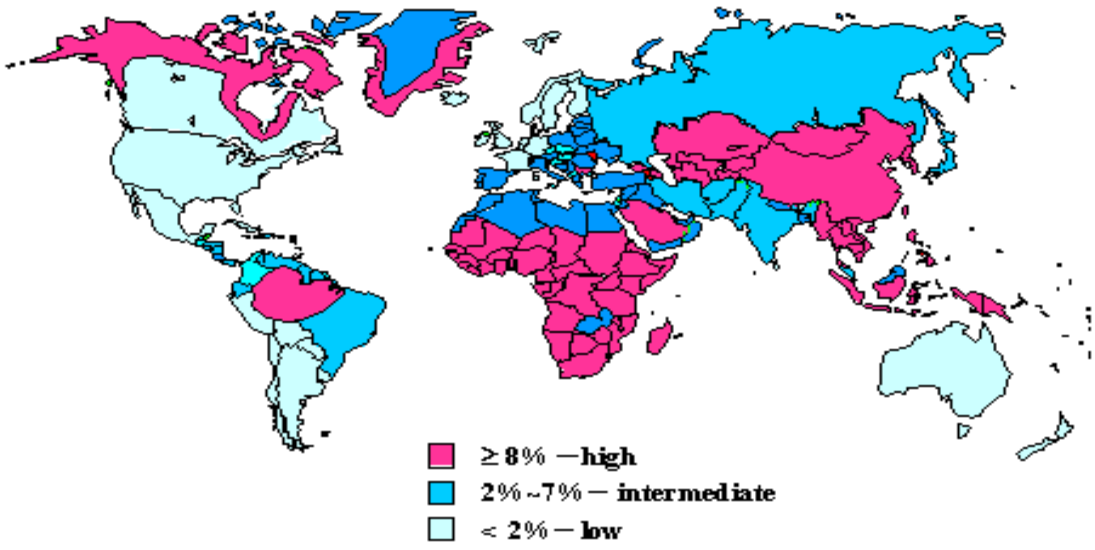

Figure 1-1: World distribution of chronic hepatitis B infection demonstrated by HBsAg prevalence.

Source of Figure: (Hou et al., 2005)

Approximately 45% of the world's population live in areas where chronic HBV infection is highly endemic; 43% live in areas that are intermediately endemic; and 12% live in areas of low endemicity. The areas of high chronic endemicity include some parts of Africa (western and sub-Saharan Africa), some parts of Asia (China, Taiwan and Republic of Korea) and the Amazon Basin (Figure 1-1).

Areas of intermediate prevalence include Japan, Middle East, East and South Europe and some parts of South America as displayed in figure1-1 above. Other areas with low prevalence are North America, North and Western Europe and New Zealand (Custer et al., 2004, Chen et al., 2000). The endemicity of chronic hepatitis B is well depended on age of individuals at time of infection and the routes of transmission. Though HBV

infection in neonates is scarce in Africa due to low number of mothers who are HBeAg positive compared to Asia, there have been rising cases of HBeAg positivity from 6.4% in 1994 to 10.5% in 2005 in parturient mothers at the Korle-Bu Teaching Hospital (KBTH) in Accra (Damale et al., 2005).

Many sub-Saharan African countries are hepatitis B endemic with carrier rates ranging from 9% to 20% (Kiire, 1996, Hyams et al., 1989). In Ghana, HBV seroprevalence was found to be 6.4% in pregnant women (Acquaye and Mingle, 1994), 6.7% in people who donate blood (Acquaye, 1991) and 15.8% in children (Martinson et al., 1996) among the general population and as high as 54.1% in patients suffering from jaundice (Acheampong, 1991). In a related research, the prevalence of HBV in patients diagnosed with cirrhosis was 42.9 % (Blankson et al., 2005) which to some extent, sheds light on Edington's work in 1957. Edington observed that, liver cirrhosis was the common liver disease causing death at autopsy a result similar to what Blankson and his colleagues got. Blankson and colleagues further found that HBV infection was significantly associated with cirrhosis and risk of development of cirrhosis in individual infected with HBV was eight fold higher compared with HCV.

1.5 Transmission

Humans serve as a reservoir of the HBV from where the virus can be transmitted from one person to another via many routes. The medium of transmission is mostly through infected body fluids such as blood, semen or vaginal fluids (Magoni et al., 2009, Adu-Sarkodie et al., 1996). Transmission via blood is the most efficient route as blood

contains high titres of the viral particles followed by wound exudates, semen, vaginal fluids with saliva containing lesser concentration of viral particles (CDC-MMWR, 1988). The virus is highly contagious and can survive outside the body for over seven days during which period it still remains infective. Perinatal and child to child transmission are major and common modes of HBV spread in areas that are highly hepatitis B endemic (Liang et al., 2009, Freitas da Motta et al., 2002).

Though perinatal transmission is common in endemic areas, in-utero transmission is quite rare. The risk of vertical transmission from perinatal infection can be as high as 70%-90% for children born to HBeAg positive mothers to about 5% -20% for children of HBeAg negative mothers (Okada et al., 1976, Beasley et al., 1977). Child to child infections usually occurs in house hold setting but could occur in day care centres due to close contact between children some of whom may be asymptomatic chronic carriers (WHO, 2001).

In developing countries, other modes of transmission of the virus include transfusion with improperly screened blood, unprotected sexual intercourse, unsafe injection practices and contact with infected body fluids such as exudates from wounds. In the west and other developed countries, the main mode of transmission is sexual contact and injection drug usage (Yokosuka and Arai, 2006). The people at most risk of contracting the infection are usually health care workers, homosexuals, children born to infected mothers, people who need blood transfusions and sexually promiscuous persons (http://*www.who.int/mediacentre/factsheets/fs204/en/*).

1.6 Structure and Morphology of HBV

Human hepatitis B virus is a prototype member of the family hepadnaviridae. It is a circular, partially double-stranded DNA virus of approximately 3,200 nucleotides long (Brooks et al., 2007). In serum, the virus consists of three morphological forms (figure 1-2).

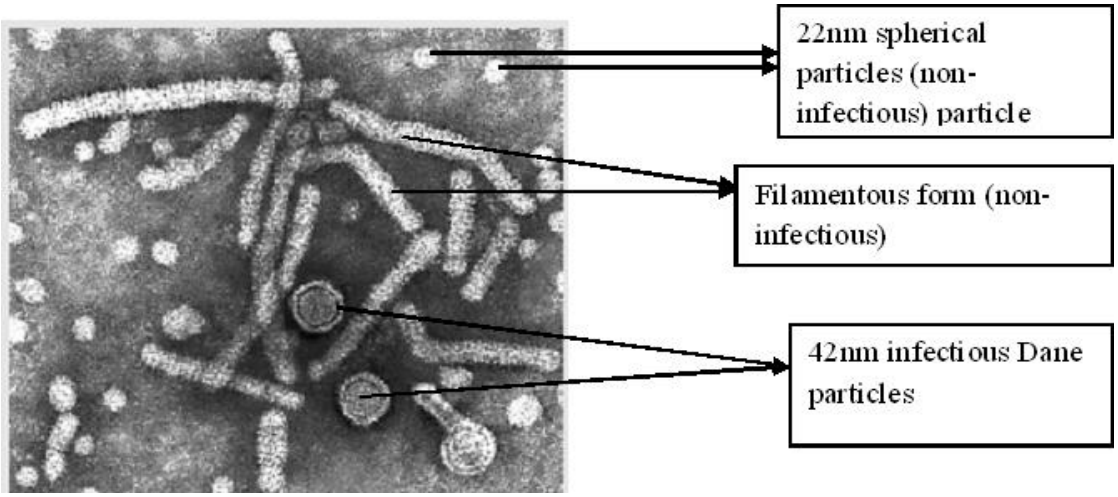

Figure 1-2: Electron micrograph of the various forms of HBV in serum.

By Prof. Linda Stannard, University of Cape Town

The lager 42nm spherical Dane particle is the infectious part of the virus. Its core contains DNA and the Dane particles are usually few in serum. The Dane particle has an envelope made of hepatitis B surface proteins surrounding the hepatitis B core proteins, at least one hepatitis B polymerase protein and the HBV genome (Onodera et al., 1982, Gerlich et al., 1982, Crowther et al., 1994). The rest of the morphological forms include a 22nm diameter particle which is spherical/pleomorphic, and the other is filamentous or cylindrical shaped (Yokosuka and Arai, 2006). The 22nm spherical form and the filamentous form also have the envelope but do not contain hepatitis B core i.e. the

genomic DNA and polymerase hence are not infectious (Onodera et al., 1982, Gerlich et al., 1982).

The envelope of all the three particles of the virus are made up of lipids that originate from the host cell endoplasmic reticula and three hepatitis B surface proteins namely the large hepatitis B surface protein (LHB), the medium hepatitis B surface proteins (MHB) and the small hepatitis B surface proteins (SHB) playing important roles in attachment and replication. These proteins are usually held together by disulphide bonds into homo and heterodimers and may be glycosylated or non-glycosylated. These are the proteins that are usually found in the serum of infected persons and picked up during testing by serology for status of a person with regards to HBsAg (Hess, 1993).

1.7 Replication

Replication mostly occur in the hepatocytes though evidence are available that the virus can replicate in the kidney and pancreatic cells, there has not been any evidence linking replication in these organs to the establishment of disease (http://ntp.niehs.nih.gov/ntp/newhomeroc/roc11/hbv_rg2public.pdf). HBV replicate through pregenomic RNA intermediate reverse transcription (Beck and Nassal, 2007) making them quite related to retroviruses but with a major difference, that is, HBV is a DNA virus while the retroviruses are RNA. It is thought that infectious virions pass through the liver sinusoid's fenestrations contained in the cytoplasmic components of the liver sinusoid endothelial cells through the space of Disse just adjacent the hepatocytes

(CDC-MMWR, 2006; IARC monographs – 100B). From there the virion interacts with other unknown specific receptors sites on the susceptible cells (hepatocytes) leading to attachment and binding by means of the PreS1 domain, subsequently penetrating the hepatocytes (Beck & Nassal, 2007, IARC monographs – 100B). It uncoat's releasing partially double-stranded relax circular DNA into the cytoplasm. This is then transported into the nucleus and cellular enzymes synthesize DNA to complete the uncompleted strand converting it to covalent closed circular DNA (ccc DNA) (Beck and Nassal, 2007). The cccDNA serves as the template for the production of HBV messenger ribosomal nucleic acids (mRNAs) including a 3.5-kb RNA pregenome. The pregenome and a viral polymerase protein (with HBV reverse transcriptase and RNase H activity) are encapsidated forming newly synthesized core particles (Butel et al., 1996, Beck and Nassal, 2007). Using the RNA pregenome as template, the reverse transcriptase synthesis the negative strand within the capsule while the RNase removes the RNA pregenome template (Butel et al., 1996).

A complementary strand to the negative strand (positive DNA strand) is synthesised but the synthesis does not proceed to completion within the core, resulting in replicative intermediates consisting of full-length minus DNA strand and a positive DNA strand of variable-length about 20% – 80% complete (Brooks et al., 2007). Core particles (nucleocapsids) containing these DNA replicative intermediates with a relaxed circular DNA may bud from pre-Golgi membranes (acquiring HBsAg in the process), exiting the cell as a virion. The nucleocapsid may re-enter the intracellular infection cycle migrating to the nucleus. There, it will deliver the freshly produced viral nucleic acid into the

nucleus of the host hepatocytes causing a great increase in the cccDNA leading to the production of more nucleocapsids (Butel et al., 1996,Beck and Nassal, 2007, Brooks et al., 2007, Tuttleman *et al.*, 1986) as demonstrated in figure1- 3 below. Amplification of cccDNA resulting in new and repetitive replication cycles produces varied hepatitis B viral genotypes, sub-genotype and strains (Norder et al., 2004, Table 1-3).

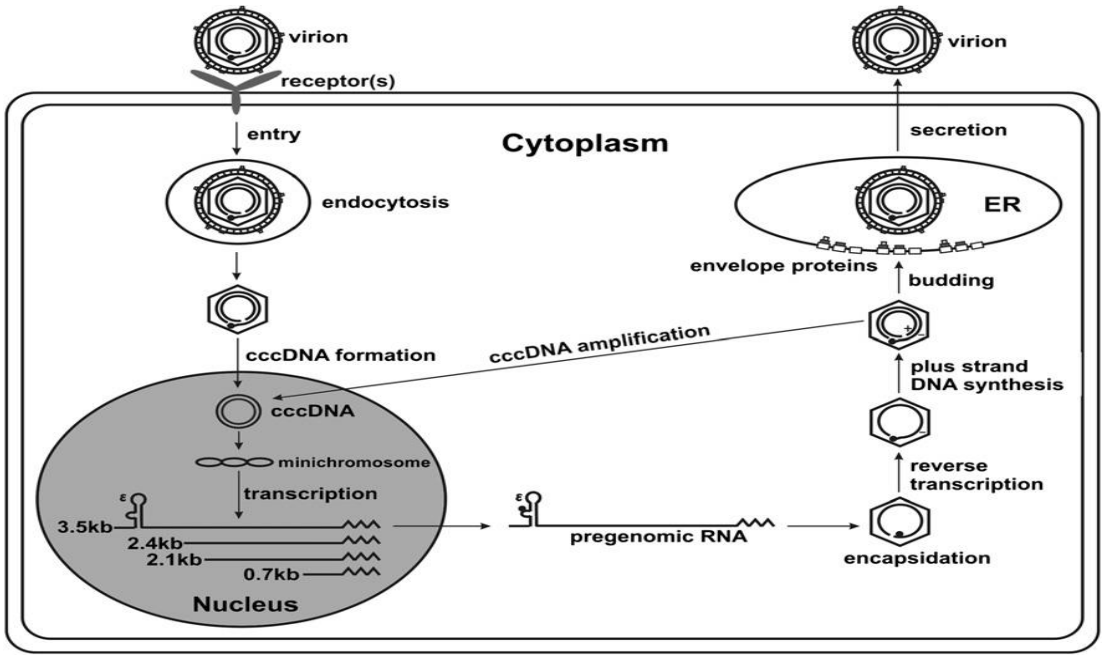

Figure 1-3: Schematic Replication cycle of HBV

Adapted from Block et al., 2007**.**

For most of the time since the discovery of HBV, its replication pattern was unknown or ignored until it was recently compared with the replication pattern of hepatitis C virus (HCV), where its irregular replication pattern was brought to the fore. While HCV immediately starts replication upon entering the hepatocytes, HBV take quite some time about 4-5 weeks after infection to show exponential replication of HBV DNA (Thimme et al., 2003, Bertoletti and Gehring, 2006).

1.8 Pathogenesis

HBV enters the body through breaks in the skin or mucous membrane and remains usually unnoticed to the innate immune system as it spreads from one hepatocyte to the other in the liver until the onset of the adaptive immune system response several weeks after infection (Chisari et al., 2010). After gaining access to the circulatory system through a bridge of the skin or mucous membrane, the virions are transported to the liver which is the preferred site to cause infection to the hepatocytes. An infected hepatocyte generally becomes large and its cytoplasm has a ground glass appearance (*http://virology-online.com/viruses/HepatitisB.htm*). The large proteins of the PreS1 domain of the proteins coat which contain amino acid of sequence 3-77 is believed to be crucial in the infection stage and may be used in binding and adsorption of the viral particle on to hepatocytes (Le Seyec et al., 1999). However some evidence suggest that receptors may be found on extra-hepatic cells such as B lymphocytes, T cell lines, monocytes and peripheral blood lymphocytes. Also, that HBV- cell interaction is mediated by interleukins, an example is interleukin 6 (Neurath et al., 1992).

Research have produced evidence that carboxypeptidase D serve as the receptors for the duck hepatitis B virus (Coffin et al., 2011), but not for hHBV even though the two viruses share a lot of similarities in their genome. The adaptive immune response is responsible for clearing viral particles and also for the pathogenesis of HBV infection (Chisari et al., 2010). The humoral immune response clears the viral particles floating in the blood system and restricting their spread, while the T cell immune response eliminates the host cells that have been infected with the virus. In acutely infected

persons when the virus is successfully cleared, the T cell response is usually vigorous, polyclonal and multispecific. It is however weak and narrowly focused in chronically infected individuals giving strong support to the assertion that HBV clearance is T cell dependent (Chisari et al., 2010).Within the hepatocytes the viruses replicate non-cytopatically within a time period of 30 to 180 days and on the average 90 days. Damage to hepatocytes is due to the adaptive immune response, mainly through virus-specific Cytotoxic T Lymphocytes (CTL) response which plays an important role in both liver pathology and viral clearance due to immune response to the viral antigens on the surfaces of infected hepatocytes (Iannacone et al., 2007, Cheruvu et al., 2007). Observations have also shown that CTL induced liver disease is enhanced by antigen-nonspecific inflammatory cells. It is also now known that platelets mediate the accumulation of CTL in hepatocytes, thereby promoting viral pathogenesis in the liver (Iannacone et al., 2007). The resolution of infections usually takes few months to some few years after acute infection. Development of chronic hepatitis B depends on the mode of transmission, the age at which infection occurs, and the immune system status of the individual at time of exposure. The pathological process that brings about cirrhosis may be carcinogenic without the direct involvement of the virus (*http://www.who.int/csr/disease/hepatitis/whocdscsrlyo20022/en/index2.htm*).

1.9 Clinical presentation

Hepatitis B may present clinically as either acute or chronic. Infection with HBV most of the time becomes subclinical in infants and perinatal infected neonates, whiles about 30% - 50% of adults and children above five years may develop clinical illness with classical

signs and symptoms of hepatitis B (CDC-MMWR, 2006, Pan and Zhang, 2005, Mahoney, 1999). Early constitutional symptoms associated with icteric hepatitis B preceding classical symptoms include malaise, fatigue and anorexia, fever of a low grade, right upper quadrant and epigastric pain lasting for one to two weeks. Patients with hyper-acute, acute and sub-acute hepatitis B may present with hepatic Porto-systemic encephalopathy and hepatic coma as a result of liver failure, somnolence, and disturbance in sleep pattern, nausea, vomiting, and jaundice. The liver enzymes such as Aspartate Aminotransferase (AST) and Alanine aminotransferase (ALT) activity become high. In some cases, skin rashes, joint pain, and arthritis may occur (Mahoney, 1999). Acute hepatitis B often resolves in one to two months. Acute hepatitis B can be detected by the presence of Anti-HBc IgM that slowly converts to Anti-HBc-IgG with recovery. Acute hepatitis B is usually characterised by presence of HBsAg, HBV DNA and HBeAg in the blood of the patient for a short period of time usually less than six months. Eventually, the antigens and DNA are cleared from circulating in the blood leading to seroconversion and protection of the individual from subsequent attacks by the virus (CDC-MMWR, 2006, McMahon et al., 1985, Ganem and Prince, 2004).

Chronic hepatitis B disease develops over a long period of time characterised by the persistence of HBsAg and HBc IgG (Mahoney, 1999) with a high concentration of aminotransferase in the serum of the patient for at least six months. These patients may appear healthy without symptoms of clinical or biochemical liver ailment and are thus called normal carriers of HBV. Some persons maybe chronically infected with hepatitis B without active viral replication. Yet some individuals may have active chronic hepatitis B where the patients have HBeAg in their serum indicating that the virus is actively

replicating with symptoms just like the acute disease. These symptoms may include fatigue, mild right upper quadrant abdominal pain and anorexia, deterioration/failure of the liver, nausea, pain or discomfort. Raised ALT can be seen in about 90% of chronically infected patients and most of these patients develop cirrhosis and stand a high risk of progressing to Hepatocellular Carcinoma (HCC). Concurrent HBV and hepatitis D infection or later super infection with hepatitis D (a defunct RNA virus) will make the disease worse compared to only HBV infection accelerating the rate of liver deterioration and progress to cirrhosis and HCC (Kayser et al., 2005).

Infection is typically asymptomatic in children aged less five years and immune suppressed adults. The overall case-fatality ratio of acute hepatitis B is approximately 1%. The possible outcomes of chronic and acute infection are shown in figure 1-4.

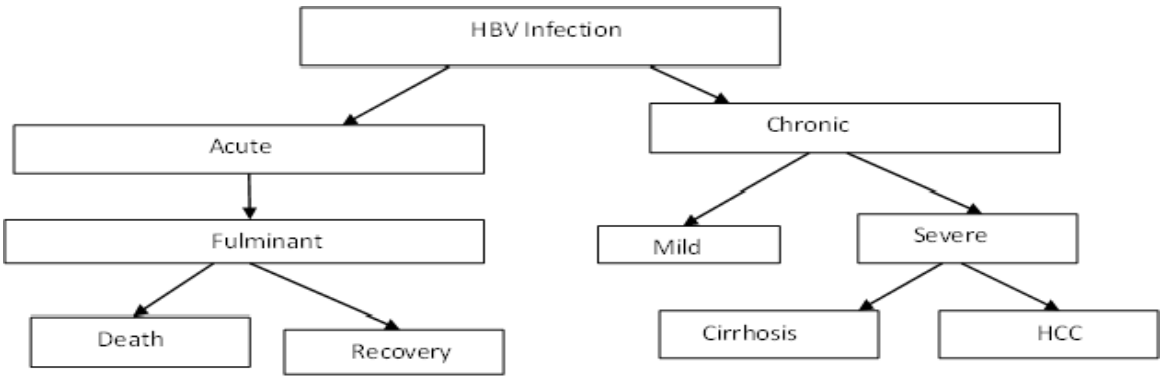

Figure 1-4: Possible schematic progress of liver disease after HBV infection in humans.

Adopted from *Chisari et al., 1997*

1.10 Diagnosis

Clinical diagnosis of hepatitis B is not based only on symptoms and physical findings because other hepatitis viruses have similar symptoms. Acute hepatitis B presents symptoms of fatigue, nausea, abdominal pain, darkening of urine, skin rashes, arthralgias etc. Later, it develops into jaundice just like the other hepatitis viral infections. Patients with chronic hepatitis B may also be quite difficult to diagnose as they often do not show symptoms or only mild nonspecific symptoms like chronic fatigue. Chronic hepatitis patients do not show many of the symptoms of acute HBV infection including jaundice until the liver damage is advance hence such patients can remain undiagnosed for a very long time; therefore, diagnosis is mainly by serology (CDC, 2006, WHO/V&B/, 2001, Mahoney, 1999).

Laboratory serological test for diagnosis of HBV infection are very sensitive and can confirm presence of viral antigens, host antibodies to the viral antigens (Mahoney, 1999) and biochemical test can detect elevated liver enzymes level (though not specific), while molecular test can detect hepatitis B virus DNA (Kayser et al., 2005). Test for the liver enzymes aminotransferases that i.e. Aspartate Aminotransferase (AST) and Alanine aminotransferase (ALT) is sensitive and one of the widely used blood tests for evaluating patients with hepatitis B. These enzymes are usually contained within the hepatocytes and are spilled into the blood stream when the liver is injured or scarred raising the levels of the enzymes in the blood indicating liver damage (CDC, MMWR, 2006).

Normal values for AST range from 5 to 40 U/L of serum whiles ALT values normally range from 5-35U/L of serum (Huang et al., 2006b) even though many laboratories have

different ranges pertaining to the area and equipment. Patients with acute hepatitis B can present very high AST and ALT levels in serum which fall to normal levels in succeeding weeks or months as the patient clears the virus and seroconvert (Kottilil et al., 2005). Patients with chronic hepatitis B however typically have normal to mild elevation of AST and ALT levels which could last for years in the immune tolerant phase of chronic infection (Pan and Zhang, 2005). Viral antigens and antibodies tested for in blood include hepatitis B surface antigen (HBsAg), surface antibodies to the surface antigen (anti-HBs), hepatitis B core antigens (HBcAg) and core antibodies (anti-HBc), hepatitis B e antigen (HBeAg) and hepatitis B e antibodies (HBeAb) (http://www.who.int/csr/disease/ hepatitis /whocdscsrlyo20022/en/index3.html#clinical). The viral antigens are transient in acute infection and are cleared from the blood stream as the patient seroconvert. Antibodies against the hepatitis B viral antigens usually can be detected in the blood within weeks of infection, and could persist without antigens in the blood for very long time thereafter and in some cases for life (Mahoney, 1999). However in chronic infection, the antigens persist for longer period of time and as the virus continuously replicate, viral progeny are released from the hepatocytes into the blood stream and their presence can be determined by measuring the viral proteins and DNA. Thus, in chronic persistent hepatitis B infection, markers to the HBV such as HBeAg, HBeAb and anti-HBs may be positive but other markers such as anti-HBc-IgG, HBsAg and viral DNA would usually remain detected in the blood (Pan and Zhang, 2005). Thus, blood tests for the antibodies as well as antigens can be helpful in diagnosing both acute and chronic viral hepatitis B infection.

1.11 Treatment

In majority of patients, acute hepatitis B is self-limiting and those cases require no treatment. Less than 1% of cases require treatment for fulminant hepatitis and symptoms such as vomiting, nausea, anorexia. In the management of chronic hepatitis B, the main target is to eliminate infectivity by stopping the replication of the viruses to prevent the infection of susceptible people and also to stop the degradation of the liver of the patient and to improve prognosis of the disease.

Hepatitis B can either be treated with antiviral drugs (nucleotide and nucleoside analogues, protein inhibitors, etc.) to stop viral replication or can be treated with immune modulators which boost the immune system to offer better resistance to the virus (Ashor, 2011). Interferons with antiviral, ant-proliferative and immunomodulatory properties are used to treat chronic hepatitis B. These interferons are to boost early maturation of B lymphocytes, enhance T helper cell activity and boost type 1 HLA expression (Malik and Lee, 2000, Lavanchy, 2004, Ashor, 2011). Some of the treatments and methods of managing hepatitis B disease is available for patients in Ghana, but they are expensive to obtain.

Many studies to find treatment for hepatitis B are in progress. In some of these studies chronic hepatitis B patients are inoculated with vaccines that have multiple antigenic components. Others are using DNA vaccines only for inoculation of the patients or a combination of the DNA vaccines and immunomodulatory cytokines or yet still others studies have the genetic constitution of antigen presenting cells directly changed

(Zuckerman, 2006). If vaccinated after exposure to HBV, the vaccine still prevent development of disease due to the delayed kinetics of the viral replication which result in delayed humoral and cellular immune response (Bertoletti and Gehring, 2006).

The onset of the HBV infection is insidious and is accompanied by the darkening of urine and pale stools. Onset of symptoms in children is more abrupt with the icteric phase being shorter. Recovery normally takes 6 to 12 weeks after the onset of illness. A very small proportion of infected individuals about 0.5% -1% presenting with acute hepatitis B develop fulminant hepatitis and consequently die from liver failure (Kayser et al., 2005). Mortality in acute HBV infection increases with age while development of chronicity reduces as age increases.

1.12 Problem statement

Hepatitis B is a vaccine preventable disease and many countries including Egypt and The Gambia have been able to reduce its prevalence through universal infant vaccination. There is very little information on chronic HBV infection in Ghanaian children. Furthermore, responses after HBV vaccination in infants in Senegal and Cameroon have suggested that country evaluation is necessary (Re-Cuille et al., 2012). In order to reduce the prevalence of HBV infections, Ghana introduced the HBV vaccine into her EPI in 2002 in line with the WHO recommendation (Van Damme et al., 1997). The prevalence of HBeAg among parturient mothers at the Korle-Bu teaching hospital was also found to rise from 6.4% in 1994 to 10.5% in 2005 (Damale et al., 2005) suggesting increase in

HBV transmission, but currently there is no policy of vaccination and administration of immunoprophylaxis of neonates from HBsAg positive mothers.

Ghana has used generic pentavalent vaccines (DPT-HepB-Hib) from different manufacturers since the inclusion of hepatitis B vaccine into the EPI. The CDC and WHO recommendation is that, when any certified vaccine is properly administered, 95% of vaccinees should produce sufficient anti-HBs to protect them. Sparse data about response of children to the HBV vaccine exist in the country, that is from the northern and middle belt of the country (Hodgson et al.,2008, Newton et al., 2010) but non in the southern sector. Consequently, the need to know the anti-HBs response of children to the HBsAg in the generic pentavalent vaccines is compelling. Also, there is no data on the anti-HBs response of children from HBsAg positive mothers who do not receive hepatitis B immune globulin and or vaccine at birth and children from HBsAg negative mothers.

1.13 Justification

The study will provide data on the pentavalent vaccine administered to children in southern Ghana. This will provide information on the immune response of children to the HBsAg in the pentavalent vaccine. It will also enable the identification of non-responders and the correlates for nonresponse. This information on the vaccination and vaccines themselves will help to reshape the policy on the vaccines and vaccination program.

1.14.1 Aim

To assess the levels of immunity to HBsAg in DPT-HepB-Hib among children who received complete doses of the pentavalent vaccine.

1.14.2 Specific objectives

- To determine the anti-HBs responses in children who received the three complete doses of pentavalent vaccine.
- To determine the HBsAg prevalence among children who receive the complete three doses of the DPT-HepB-Hib
- To determine correlates for anti-HBs responses.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The National Expanded Program on Immunization (EPI) Policy

In 1992, the World Health Assembly approved the target set by WHO to integrate universal hepatitis B vaccination into the childhood vaccination programs of highly endemic countries (with greater than 8% hepatitis B Prevalence) by 1995 and to other countries with low endemicity by1997 (www.who.int/**vaccines**-documents/DoxNews/updates/updat31e pdf). As such Ghana sought funding from Global Alliance for Vaccines and Immunization (GAVI) and started universal infant vaccination in 2002 (Ghana EPI profile, 2002-2005).

Ghana fully integrated the hepatitis B vaccine into the routine immunization program, providing free vaccine for all infants at 6, 10 and 14 weeks of age which is the normal vaccination schedule for the Diphtheria Pertussis and Tetanus (DPT) combination of vaccines for children. This schedule is adopted for children of many sub-Saharan African countries based on the low numbers of HBeAg negative mothers (Ekra et al., 2008). This integration process is aimed at saving cost and to cover as many of the children as possible. The vaccination programme is a collaborative project between the Ghanaian Ministry of Health and GAVI for the vaccines to be made available for all children during child welfare clinic attendance. In 2002, Ghana replaced DPT combined vaccine in the EPI scheme with the pentavalent vaccine containing Diphtheria, pertussis, Tetanus Hepatitis B, *Heamophilus influenzae* type b (DPT-HepB-Hib) antigens, with the objective to universally vaccinate all infants and children against Hepatitis B and *Haemophilus*

influenzae type b (GHS report 2001). The national Hepatitis prevention and control program also has as an objective, to reduce morbidity and mortality caused by Hepatitis B through the incorporation of Hepatitis B vaccine into EPI in addition to screening blood for transfusion as part of the hepatitis B infection control (Ghana EPI profile 2002-2005). When administered, the vaccine prevents the development of the disease after infection with HBV from mother to child, from one child to the other and from the general public to the child. The only study relating to the HBsAg in the pentavalent vaccine in Ghana was a trial to determine the influence of vitamin A supplement on the antibody response of infants to the HBsAg. This study showed no significant difference between those who took the vitamin A supplement and those who did not take the supplement (Newton et al., 2007).

2.2 Types of Vaccines

Hepatitis B is a vaccine preventable disease and needs concerted efforts in national immunization program to eliminate the disease, because of the high disease burden and the large pool of chronic carriers most of whom are asymptomatic (Van Damme et al., 1997). A vaccine against hepatitis B was developed in 1969 by Dr Blumberg two years after discovering the virus (http://www.hepb.org/about/blumberg.htm) and the vaccine has been available for use by the general public since 1982 (Zanetti et al., 2007); however, cost of vaccine has prevented many people at high risk from getting the vaccine. The development of this highly effective vaccine, the first vaccine to prevent human cancer represented a great achievement in medicine in the 20th century. There are two types of vaccines produced for the prevention of hepatitis B disease. These are the

plasma derived vaccines and recombinant DNA vaccines: The plasma derived vaccine was the first type of HBV vaccines to be produced in the early 1980s from the collection of plasma from chronically infected individuals. The vaccine was then produced by purifying, formalinating and or heat–inactivating and adsorbing the HBV particles on alum which also serve as an adjuvant. The 22nm spherical and filamentous forms of HBV do not contain any nucleic acid and therefore non-infectious and are therefore used for the production of the vaccine *(http://www.who.int/csr/disease/hepatitis/ whocdscsrlyo 20022/en/index4.html#vaccines)*. The treatment given to the plasma is to ensure that other infectious pathogens such as HIV that may be present in the plasma are destroyed and not transferred to the recipients of the vaccine (*www.who.int/**vaccines**-documents/DoxNews /updates/ updat31e pdf*). This gave the plasma derived vaccines long production life span. The plasma derived vaccines constituted the first generation of the hepatitis B vaccines (Zuckerman, 2006).

The recombinant DNA vaccines were introduced in the middle of 1980s. The S gene of the virion was cloned, isolated and inserted into an expression vector – yeast (*Saccharomyces cerevisiae*) or Mammalian Chinese hamster Ovary (CHO). The vector then expressed the proteins which are assembled into the 22nm spherical and the filamentous sub forms of the HBV. This type of vaccine has a short production life cycle of 12 weeks compared to the plasma-derived vaccine with production life span of 64 weeks, and these vaccines constituted the second generation of HBV vaccines.

Third generation recombinant vaccines have being produced from the S, pre-S1and pre-S2 gene products that closely looks and function like the major proteins of the HBV coat and therefore highly immunogenic. This generation of vaccines has overcome the non-responsiveness of the second generation vaccines with product from only the S component of the HBsAg (Zuckerman, 2006). Several recombinant yeast derived vaccines are produced by various companies e.g. EngerixB^{R} (Smithkline Beecham, 1992), Recombivax HB^{R} (Merck & Co). These vaccines produce their main humoral response by stimulating T helper cells and B cells specific to the HBsAg to produce neutralizing antibodies to the "a" determinant of the HBsAg within weeks of vaccine administration (Zuckerman, 2006). Hepatitis B vaccine is 95% effective in preventing HBV infection and its chronic consequences when appropriately administered (www.who.int/**vaccines**-documents/DoxNews/updates/updat31e). Four doses of plasma derived vaccine administered to children were 84% effective against infection and 94% effective against chronic carriage in The Gambia vaccination programme (Fortuin et al., 1993). Seroprotection of children who received four doses of hepatitis B vaccine in Ivory Coast was up to 85.5% of the study participants showing the immunogenicity of HBV vaccines (Magoni et al., 2009). The vaccine currently administered by the ministry of health's EPI is the Quinvaxem®inj. (DPT-HepB-Hib fully liquid combined vaccine) manufactured by Berne Biotech Korea Corporation, a Crucell Berne Switzerland subsidiary in Korea.

The vaccine batch numbers received by EPI and distributed among health facilities include 451620 and 451621 by Berne Biotech, Korea in August, 2011. In April 2011, another batch 1452021 was received. In June 2011, another set of vaccines with batch

number P1033/SBP (Panacea biotech) India, was received but this batch was out of stock in the EPI stores at the time of sample collection. However, there was small quantity of vaccine UVA0006 Korea, (LG Life) on request for mothers only.

2.3 Vaccination

This is usually done before individuals are exposed to the virus or could also be done in addition to administration of an immune globulin immediately after an individual is exposed to the virus. Safe genetically engineered vaccines are available for vaccination of people at risk.

2.3.1 Pre Exposure

Many vaccine schedules are in use depending on the prevalence of the virus in the area. The best schedules for a particular country or situation is the schedule that will enable the completion of the vaccine series. Ghana has adopted and integrated the vaccine into the EPI together with the DPT combination of vaccines and administers the vaccine at 6, 10 and 14 weeks of age (GHS report 2002, Aspinall and Kocks, 1998); however, no provision has been made for a booster dose. This schedule is also practiced because of the fact that HBeAg carriage among pregnant women is low in Africa (Ekra et al., 2008), partly to reduce the number of needle pricks to children and also to reduce number of visits of mothers and their children to the health facility. Other countries such as The Gambia and Ivory Coast, which has been vaccinating children since the early 1980s and 2000 respectively use four doses (Fortuin et al., 1993, Magoni et al., 2009). Various schedules for administering the hepatitis B vaccine to children are shown below in table

1. Schedules I and II give a birth dose but schedule I give additional dose to sum up to a total four while schedule II gives only three doses just like schedule III. Schedule III however, start at six weeks of baby's age and this is the schedule currently used in Ghana. With the exception of the birth dose which should be a monovalent vaccine dose, the other doses can be given as combined vaccines (www.cdc.gov/vaccines/pubs/ pink book/hepb).

Table 2-1: Option of HBV vaccine schedules for infants

						Hepatitis b vaccine schedule		
						With dose at birth		No dose at birth
Age	**visit**	**Other vaccines**				I	II	III
0 Week	At birth	BCG, OPV				HepB – birth	HepB - birth	
6 Weeks	1		OPV	DPT		HepB	HepB	HepB
10 Weeks	2		OPV	DPT		HepB		HepB
14 Weeks	3		OPV	DPT		HepB	HepB	HepB
9-12 Months	4				Measles			

Adopted from department of vaccine, WHO, 2001 (WHO/V&B/01.31, www.who.int/vaccines-documents /).

About 5-10% of vaccinated people do not mount immune response to the vaccine after the first complete three doses, and of these about 62% -98% of them respond after a second round of three doses. Other proposed pre exposure schedules include 2, 4, 6 months of age and 1, 6 months of aged (Greenberg et al., 1996, Da Villa et al., 1997).

2.3.2 Post Exposure.

Mothers who are HBsAg positive and particularly HBeAg positive expose their children to the virus during birth; hence, such children need to be given post exposure prophylaxis immediately after birth (WHO, 2001,WHO/V&B/01.31, www.who.int/ vaccines-documents/). Post exposure prophylaxis involves the administration of hepatitis B vaccine with or without HBIG. A high antigen dose per volume of the vaccine is preferred because of the high probability of inducing seroconversion with high antigen vaccine dose. While higher concentration of antigen per vaccine dose may not require HBIG to illicit a good immune response, low antigen dose hepatitis B vaccine will require HBIG to give a good immune response in neonates (Andre and Zuckerman, 1994).

2.4 Hepatitis B Viral Markers

The progression of HBV infection is associated with different viral markers in blood and indicates particular stages of infection. These include the surface, core, and e antigen markers and their corresponding antibodies.

2.4.1 The hepatitis B surface antigen

This is the major component of the coat of the hepatitis B viral particle, coded for by the S gene weighing about 24 kilo Daltons and may or may not be glycosylated. The product of the S gene constitutes the major protein of HBsAg. Products of the PreS1 and PreS2 incorporated into the HBsAg are in small quantities in the shells of the non-infectious HBV particles (Mahoney, 1999). Nine antigenic subtypes have been identified by

serology but four of the subtypes have been identified as the main subtypes. All the antigenic subtypes have a common epitope /determinant "a" and one each of two mutually exclusive sub-antigenic determinant types "d/y or w/r". The combination of the common determinant "a" and the other mutually exclusive epitopes vary in geographical distribution with some subtypes predominating in some areas and some categories or ethnic groups of people (Franco et al., 2012, Fagan and Williams, 1986). Subtype adw prevails in Africa and ayw in West Africa (Zuckerman, 2006).

2.4.2 Hepatitis B core antigen

This is a component of the nucleocapsid core antigen system, essential for the packaging of the virus and coded for by the C gene (Eligouhari et al., 2008) in the L- strand and weighing 18-19 *k*D. When transcribed, it usually moves into the endoplasmic reticulum where it gets cleaved. It is usually difficult to locate this antigen in serum by conventional methods but can be easily identified in liver biopsy of patients where it appears in the nuclei of infected hepatocytes (Fagan & Williams, 1986, Mahoney, 1999).

2.4.3 Hepatitis B e antigen

This antigen is coded for by the C gene (Eligouhari et al., 2008) and translated from the same gene as HBcAg weighing 15.5 *k*D. This is a soluble antigen in the blood created by the pre-mature proteolytic cleavage of the core antigen. The presence of this antigen indicates that the virus is rapidly replicating in the patient and thus high numbers of virions are present in the blood; hence, the patient at this stage is highly infectious. This antigen is usually present when the infection is acute or chronically active but is not essential for viral replication (Mahoney, 1999, Fagan and Williams, 1986).

2.4.4 Hepatitis B surface antibody

This is a protecting and neutralizing antibody produced in response to infection with the HBsAg (Mahoney, 1999). The presence of this antibody against the common epitope determinant "a" in an infection of one subtype confers immunity against re-infection or new infections with other subtypes (Fagan and Williams, 1986). When this antibody develops after the HBsAg has been cleared from the serum at about six months after primary or acute infection, the antibodies persist for life (Mahoney, 1999). However, a long time after infection their quantities may be so low that some test regimes may not be able to pick them up. Even then, there is still immunological memory that will enable the person's immune system to appropriately respond in time of infection. The titre of this antibody in the blood is measured in milli-international units per milli-litre of blood (mIU/ml or IU/L) and 10 mIU/ml or 10IU/L is the minimum amount of anti-HBs needed for protection (Van Damme and Van Herck, 2007, http://www.cdc.gov/Vaccines/ pubs/ pinkbook/downloads/hepb).

2.4.5 Hepatitis B core antibody

This antibody is produced by the body in response to infection with the Dane particle and can be detected just after the appearance of HBsAg. Detection of the hepatitis B core Immune globulin M (HBc IgM) is an indication of acute infection with Dane particle but the absence of anti-HBc-IgM and the presence of hepatitis B core Immune globulin G (HBc-IgG) together with HBsAg is an indication of chronic infection. Persons with antibodies due to vaccination have no anti-HBc. The anti-HBc may usually be the only marker present in the serum of an infected person during the window period when the

individual has cleared the HBsAg and HBeAg and about to seroconvert. The anti-HBc persists for life (Fagan and Williams, 1986, http://www.cdc.gov/Vaccines/pubs/pinkbook/ downloads/ hepb.pdf). This antibody has been found in the sera of people who have been vaccinated against HBV. In Ivory Coast, Magoni et al., 2009 found that 5.2% of sampled population had anti-HBc and else-where Yen-Hsuan et al., 2001 also found different percentage prevalence in different groups of research participants.

2.4.6 Hepatitis B e antibody

This marker develops after HBeAg a soluble protein in the core has been cleared from the blood of infected person. The presence of HBeAb in the blood of persons infected with HBV indicates that the person in less infective; thus, the number of virions in the blood are fewer.

2.5 Immune Response to HBV Vaccine

Innate immunity generally plays a role immediately after infection to halt the invasion and colonization of body tissues by HBV and begin the process of developing appropriate adaptive immune response leading to the production of type 1 interferon and the activation of natural killer (NK) cells (Bertoletti and Gehring, 2006).

HBV is not directly cytopathic to hepatocytes; rather, injury to hepatocytes as a result of HBV infection is through immune response to viral antigens presenting on the surface of hepatocytes, with the main target of immune response being HBV core antigen (Huang et

al., 2006a, Baumert et al., 2007).The main immune response of individuals who receive the vaccine is against the epitope “a” which is common to all subtypes of the hepatitis B genome and this results in protection against all subtypes (Zuckerman, 2006). HBV infection indirectly depends on several factors that can influence the immune response, such as age at which infection occur (Pan and Zhang, 2005), host genetic factors e.g. major histocompatibility complex (MHC) which is inherited in dominant Mendelian fashion, abnormal haplotype or the absence of immune response gene for HBsAg (Kruskall et al., 1992, Huang et al., 2006a). The genetic variability of the virus due to the different genotypes and subtypes available has a direct impact on viral gene expression and consequently infection of host cells (Norder et al., 2004). These factors promote non response leaving the person who might have been vaccinated still susceptible to infection by HBV.

The responses of various individuals to the vaccine are varied and the definition of persons who receive the full dose of vaccine as a non-responsive, hypo-responsive or hyper-responsive person depends on the antibodies titres to HBV produced by the person. However, antibody levels < 10 mIU/ml, < 100 mIU/ml and >100 mIU/ml produced between one to four months after administration of full vaccine dose characterises non-responsive, hypo-responsive and hyper-responsive persons respectively (Zuckerman, 2006). Many children who were vaccinated at birth loss their anti-HBs by age five (Petersen et al., 2004), while another study argued that protection was shown to be adequate after 25 years of vaccination (Van Damme and Van Herck, 2007). About 14% of children aged between 5-12 months were non responsive in a study on the antibody response of children to the standard three vaccine doses of hepatitis B in Iran. Males

constituted 2.2% of those who were non-responsive and they had hepatitis B core antibody, an indication that they were infected with the wild type virus either before or after vaccination (Moradi et al., 2009). It was also evident from this study that, males were more likely not to respond than females. Yet another study in Iran produced a non-response rate of 12.4% (Esmaili and Seyedkolal, 2003). Elsewhere in India and Sweden, a response rate of 100% was obtained in various studies (Sood et al., 2002, Harrison et al., 1991). In Mahmoud city, 81% of children aged 12-16 months responded to a Cuban-made vaccine (Azarkar, 2004).

Anti-HBs produced due to vaccination with recombinant DNA vaccines has a 62.7% rate of disappearance over a 12- 15 years period compared to plasma derived hepatitis B vaccine though the DNA recombinant vaccines offer better anamnestic response following booster vaccine doses (Kao et al., 2009, Fallahian and Zamani, 2010). Bivariate analyses of data from a study on immune response of health care workers to hepatitis B vaccine indicate higher level of non-responsiveness in males than in females i.e. 18% and 8% respectively. Multivariate analysis in this same study also shows reduced seroconversion rate in males compared to females. But overall, 14% of subjects remained non-responsive with serum anti HBs titres below 10 mIU/ml (Zeeshan et al., 2007). In a mass vaccination with Cuban hepatitis B vaccine in Iran, 15.6% of children who received three doses of the vaccine had anti HBs titres of less than 10 mIU/ml while just 27.7% were hypo-responsive (Dahifar, 2004). A study to determine if children (infants) born to anti-HBs positive mothers had any degree of impairment in their response to the hepatitis B vaccine due to acquired maternal antibodies showed no significant difference in response between children of Anti- HBs positive mothers and

Anti-HBs negative mothers one month after full dose of three shots of vaccine administration (Hu et al., 2008). However, in an unusual response rate in Mongolia, only 17% of vaccinated children had anti-HBs levels more than 10 mIU/ml (Fallahian and Zamani, 2010).

About 5%-10% of all immunocompetent vaccinees will not mount immune response to the hepatitis B vaccine after administration, and this is caused by several factors that adversely affect the antibody response to HBsAg, including HLA-DR alleles, the site and route of administration (lower for those who receive vaccine at the buttocks), sex, advancing age, impairment of T helper cell response, other factors such as smoking, nutritional status, and genetic factors of vaccines, body mass (overweight), immunosuppression, and immunodeficiency (Zuckerman, 2006, Fallahian and Zamani, 2010). These factors could contribute a great deal to reduced immune response, but the mechanisms underlying non-responsiveness to the S component of hepatitis B surface antigen in humans remain to a large extent unexplained (Zuckerman et al., 1997).

An unexpected low response of 54% was realized in healthy vaccinees who received the vaccine at their buttocks in health care workers whiles the response to the same vaccine in other health care workers in centres elsewhere was higher when the vaccine was administered at the deltoid (Zuckerman, 2006). However, evidence is accumulating that different HLA-DR3, DR7; DQ2 alleles including the absence of HLA-A2 alleles are associated with specific low responsiveness in different ethnic populations. Considerable experimental evidence is available that the ability to produce antibody in response to

specific protein antigens is controlled by dominant autosomal class II genes of the major histocompatibility complex in mice (Zuckerman et al., 1997, Kruskall et al., 1992). Much effort has been devoted to overcoming class II linked non-responsiveness to current hepatitis B vaccines (Zuckerman et al., 1997, Milich et al., 1985).

The pre-S1 and pre-S2 domains play a significant immunogenic role in augmenting hepatitis B surface antibody responses, preventing the attachment of the virus to hepatocytes and eliciting antibodies that are effective in neutralising viruses. This stimulates cellular immune response, and avoids immune non-responsiveness to the S gene (Zuckerman et al., 1997, Milich et al., 1986).

Thus, several studies indicate that pre-S components should be included in new recombinant or synthetic vaccines. For example, the pre-S2 region was found to be more immunogenic to T and B cells than the S regions in mice (Milich et al., 1985, Klinkert et al., 1986). Ferrari et al., 1989, suggested that the Pre-S1 could function as a strong T cell immunogen in humans. These studies led to the development of triple antigen vaccines like Hepacare 1,containing proteins from the S region (S, PreS1 and PreS2) of the HBsAg which is highly immunogenic compared to conventional yeast derived vaccines (Zuckerman, 2006, Yap et al., 1995).

2.6 Serological Events after Infection

The serologic events in HBV infection are indicated by HBV markers which appear with time after HBV infection and may be the antigens of the virus or antibodies produced by the host in response to the antigens. Some differences exist depending on whether the

infection is acute or chronic. Hepatitis B surface antigen, Viral DNA, HBV DNA polymerase activities, anti-HBc-IgM and HBeAg can be detected in serum early in the incubation period. HBsAg is the first marker to appear within 1 and 10 weeks after infection alongside HBeAg or immediately after the detection of HBsAg. By this time, viral DNA and viral DNA polymerase can also be detected in serum, constituting the viremic phase. Anti-HBc is the first of many HBV antibodies that show in the blood of the patient just at about the time HBsAg appears in the blood as shown in figure 2-1 below (Guerrant et al., 1999, Ganem and Prince, 2004). High numbers of viral particles up to 10^9-10^{10}IU /L are present in the serum of the patient when the HBeAg is positive in a test and at this period the person is highly infectious (Ribeiro et al., 2002). Symptoms appear amidst high titres of anti-HBc-IgM after one to eight weeks when infection is acute and may last up to 24 weeks or more after infection and at the onset of clinical illness. HBsAg typically disappears between the sixth to ninth months after being exposed to HBV in acute infections. In majority of adult, few children and very few neonate/infant infections, the immune system fights the HBeAg and begins HBeAg clearance from the blood by the third month leading to the production of HBeAb as in figure 2-1 below. Gradually, HBsAg disappears and is replaced by anti-HBs, whiles HBcAg is replaced by anti-HBc-IgG indicating the resolution of the infection (Zuckerman, 2006). The various serological markers and their course of development in the natural history of acute infection are summarized below in figure 2-1. Anti-HBc may be present during the window period when HBsAg and HBeAg have been cleared and anti-HBs and HBeAb are formed.

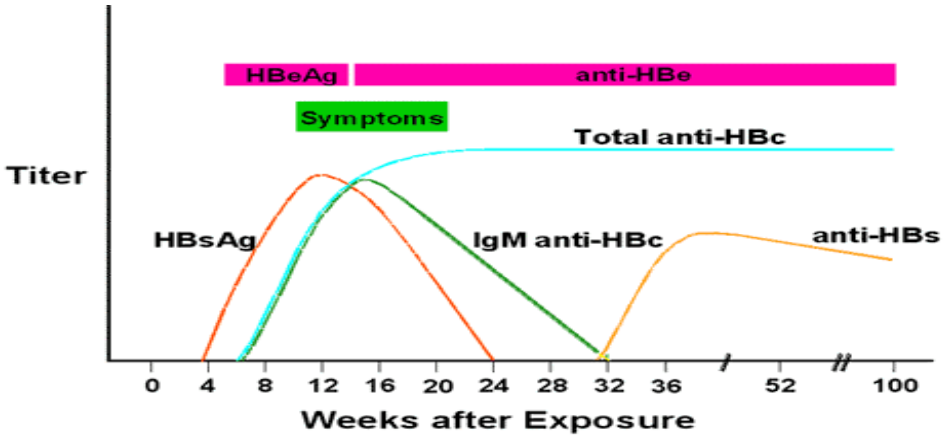

Figure 2-1: Development in serological markers in acute HBV infection.

Source: CDC

Infection becomes chronic in about 90% of infants/neonates who are perinatally infected, about 30% of children infected before they are six years of age and 3-5% of older children and adults become chronically infected when exposed to HBV. In these cases, HBsAg remains positive for more than six months with or without HBeAg. The mark of chronic infection is that no anti-HBc IgM, rather anti-HBc-IgG is detected in serum, and HBV DNA remains low in serum of the infected individual after six months and in some cases for life (Ganem and Prince, 2004). The series of events in chronic HBV infection are summarized in figure 2-2 below.

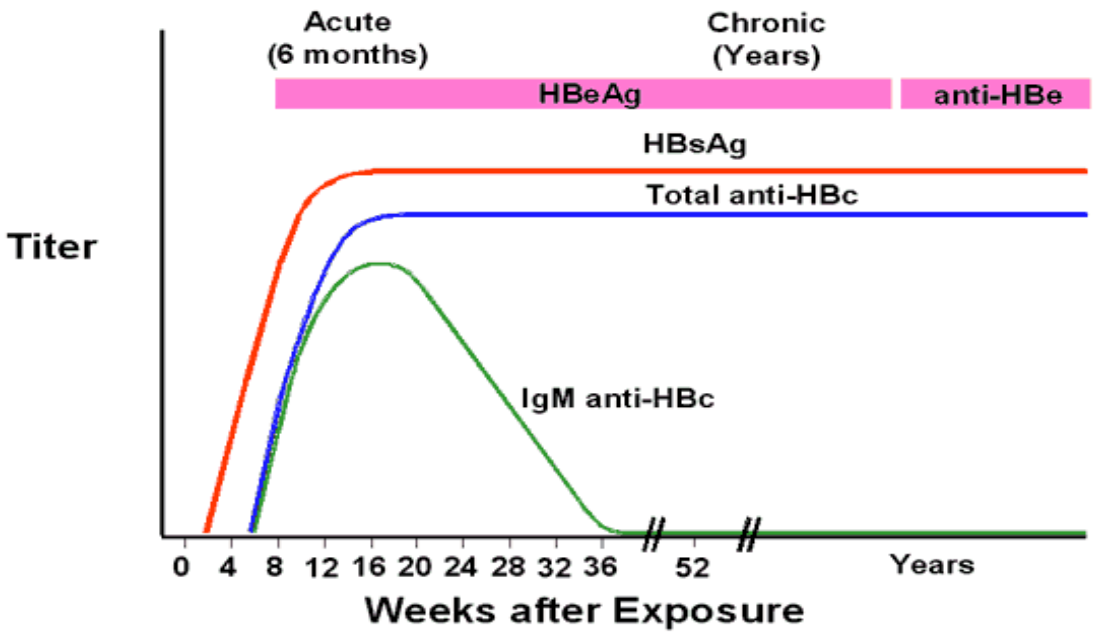

Figure 2-2: Progress of serological markers in chronic HBV infection

Source: CDC

2.6.1 Developmental stages of HBV infection

Developments that occur after HBV infection can be divided in to four distinct stages including the immune tolerant phase, the immune clearance phase, the inactive immune carrier phase that could be with or without reactivation, and the immune phase.

In the immune tolerant phase following acute infection, the infected individual shows HBeAg in serum with high levels of HBV nucleic acids. However, very little to no signs and symptoms, low to no sign of hepatocyte histological activity and low aminotransferase is detected in serum (Pan and Zhang, 2005). This stage hardly progress into cirrhosis but may develop into the second stage when the tolerance to the HBV is lost. Loss of immune tolerance results in the lysis of the infected hepatocytes and this

action is mediated by the immune system (Lee, 1997). This stage is characteristic of infection in children who were perinatally infected and some young adults. This stage of infection could last for 2-4 weeks but sometimes for years (Merican et al., 2000). The presence of HBeAg makes this kind of patients highly infectious and can easily transmit the virus to susceptible people they come in contact with.

The second phase of infection is characterized by the seroconversion of HBeAg to HBeAb with a consequent decrease in the concentration of viral particles in serum of infected individual. Serum aminotransferases level rises due to immune mediated lysing of infected hepatocytes. When the immune system is competent and efficient, the phase may last for 3-4 weeks otherwise, it could take years for the immune clearance phase to be over. This phase would usually occur between the ages 15-35 years especially when children are infected at an early age or perinatally. Severe chronic hepatitis may occur histologically, with patient prognosis depending largely on the severity/extent of damage to hepatocytes and the duration within which the damage occur (Pan and Zhang, 2005, Merican et al., 2000).

In the inactive carrier phase with or without reactivation, most of the infected hepatocytes are cleared and the process is mediated by the immune system, leading to seroconversion i.e. HBeAg is converted to HBeAb. At this stage, active viral replication is halted even though HBsAg can still be detected in the serum while serum aminotransferases and HBV nucleic acid levels decline (Merican et al., 2000). Some of these carriers can sometimes experience immunosuppressed induced chronic hepatitis characterized by

elevated levels of HBV DNA and aminotransferases, and may or may not seroconvert and showing mild to severe liver histological activity (Pan and Zhang, 2005).

Finally, some patients proceed to the fourth phase – the immune phase, where all HBsAg are cleared from the serum and anti-HBs produced to replace them. Also, HBV nucleic acids are no longer detectable in serum, and there is no risk of reactivation of replication stage. The production of anti-HBs signals the development of lifelong immunity against the HBV (Merican et al., 2000).

2.7 Liver Cirrhosis and Hepatitis B Virus

Studies have shown a positive correlation between hepatitis B infection and liver cirrhosis which is an end stage and a chronic irreversible disease of the liver that affects mostly people living in hepatitis B endemic areas. Going by the natural history of hepatitis B infection and its consequence, 10%-33% of people who get infected become persistently infected and about one-quarter to one-half of them will have their conditions degenerating to cirrhosis (Blankson et al., 2005). Studies by Edington in 1957 and from unpublished information from the Pathology Department, Korle-Bu teaching Hospital indicate liver cirrhosis is the leading cause of death from liver diseases in Accra (Edington, 1957, Blankson et al., 2005). Blankson et al established a strong association between liver cirrhosis leading to death of patients and HBV infection in Accra.

2.8 Hepatitis B Virus and Hepatocellular Carcinoma (HCC)

Chronic HBV infection is a risk factor for developing HCC and those at high risk are adult males and chronic hepatitis B patients presenting cirrhosis. HBV carriers show 98 fold increase risk of developing HCC as compared to their non-carrier counterparts (Venook et al., 2010). The risk is much greater for those carriers who are HBeAg positive even though the carriers with HBeAb also has substantial risk of reverting to HBeAg positive state and developing HCC (Lavanchy, 2004, Ganem and Prince, 2004). About 15% -40% of chronic HBV patients will usually develop HCC, liver cirrhosis, or liver failure (Lavanchy, 2004). Just about 5% of patients with cirrhosis develop HCC, while about 60% -90% of HCC patients will usually have underlying cirrhosis (Remis et al., 2000). There is no specific oncogene sequence that is known to be responsible for the tendency of patients with cirrhosis to progress to HCC. The tumour responsible for HBV related HCC develop from chronic liver inflammation and repeated regeneration of hepatocytes after several years (about 25-30 years) of HBV infection (Mahoney, 1999, Lee, 1997). HCC is ranked 7th and 9th most common cancer in males and females respectively worldwide with over 500, 000 people dying annually due to HCC of which the male to female ratio is 4:1. The incidence of HCC varies with geographical location, race, sex and age (*http://www.who.int/csr/disease/hepatitis/whocdscsrlyo20022 /en/ index2.htm*).

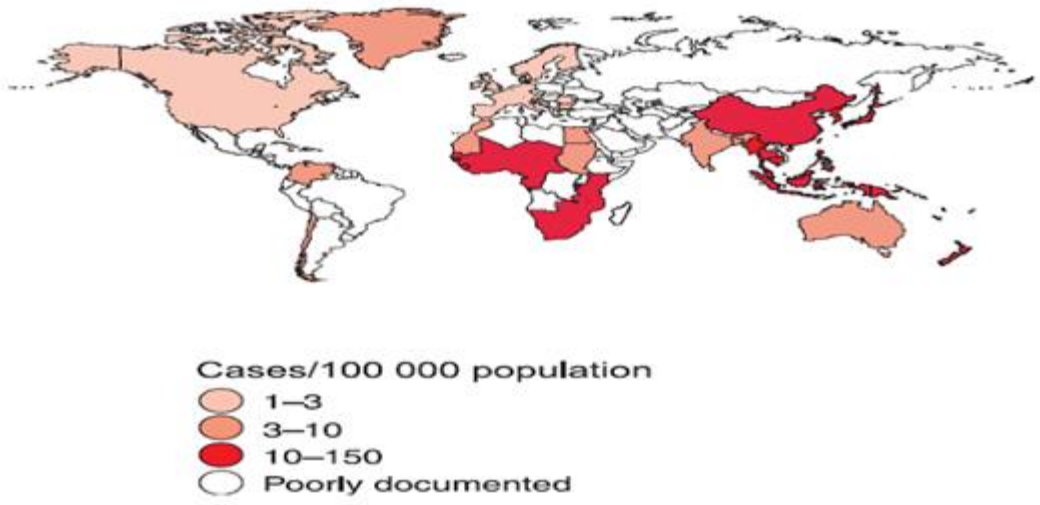

Figure 2-3: Annual incidence of Hepatocellular carcinoma.

Adopted from Lavanchy, 2004.

Primary liver cancers in the world are mostly caused by HBV accounting for 60%-80% of these cancers (Remis et al., 2000). Regions with high HBV prevalence such as sub-Saharan Africa, East and South–East Asia also show a high rate of HCC incidence of about 10-150/100 000 people annually see figure 2-3 and it is one of the three main causes of death in these areas (Lavanchy, 2004). HCC is fatal and patients with this condition have median survival frequency of less than three months. However, when the condition is detected early it can be treated. Hope was however regained with the development of the hepatitis B vaccine and the initiation of universal vaccination of children in the public. With the institution of the universal vaccination of children and infants, some countries have seen significant reduction in HCC. The incidence of HCC in children 6-14 years of age in Taiwan declined from 0.7 per 100, 000 to 0.36 per 100,000

from 1981 to 1994 with an associated decline in mortality rate due to HCC (Chang et al., 1997).

2.9 The Results of Hepatitis B Viral Vaccines Administered in other places

Upon the introduction of the hepatitis B vaccine into the regular EPI programs many countries have experienced a significant drop in the prevalence of the HBsAg among the fully vaccinated children who have either received three or four doses of the vaccine depending on the vaccine's immunogenicity as stated by the manufacturer or schedule of vaccination. In a study of HBsAg seropositivity among 1000 children who were fully vaccinated, only 0.8% of them showed the surface antigen while in the control group of 500 non-vaccinated children, 2.2% were HBsAg seropositive (Reda et al., 2003). Almost 88% of children who had complete three doses of vaccine either on regular or irregular intervals had protective levels of anti-HBs >10 IU/L in Babol Amir kola, Iran. 28.8% of the participants were hypo-responsive with Anti-HBs between 10 and 100 IU/L while 58.8% were hyper-responsive, having Anti-HBs > 100 IU/L (Esmaili and Seyedkolal, 2003).

Carrier rate of HBsAg also significantly differed in Senegal between vaccinated and non-vaccinated children, that is 2% versus 19%, respectively (Coursaget et al., 1994). A follow up study in Italy over almost 20 year period from 1978 to 1997 showed a decrease in prevalence of HBsAg from 13.4% to 3.7% in the general population, and 6.8% to 0.7% in children and adolescents (Da Villa et al., 1998). A 15 year follow up study in China also showed 8.2% chronic HBsAg carriers in non-vaccinated cohort compared to 0.3% in vaccinated cohort (Liao et al., 1999). Elsewhere in Saudi Arabia, the overall prevalence

of HBsAg among vaccinated children in the general population dropped from 6.7% to 0.3% (Al-Faleh et al., 1999). The western sub-Saharan Africa showed very high age-specific HBsAg prevalence which reached 12% in the age group of 0 to 19 years of age in 1990. However, there was a decrease in the prevalence in 2005 though the western sub-Saharan Africa still showed the highest endemicity which was more pronounced in males (Ott et al., 2012a).

Also, in a review of the work done on hepatitis B, prevalence of HBsAg among children aged 0-14 years increased in southern sub-Saharan Africa in 2005 compared to 1990 with younger females having prevalence of about 8-9%. In eastern sub-Saharan Africa, there was an increase in HBsAg prevalence in the younger ages while the prevalence was static in other age groups and this rose to a peak of 7% in 2005 in young boys and girls of 0-4 years; however, this prevalence decrease with increase in age. There was a decrease in prevalence of HBsAg in central sub-Saharan Africa which was a high endemic zone in the 1990 but became intermediately endemic in 2005 across all ages (Ott et al., 2012a).

In North Africa, there was a decrease in HBsAg endemicity across all ages from 1990 to 2005 especially in young males, and this is because these countries started the universal vaccination of children in the early 1990s (Mansour et al., 1993). The high endemicity of HBV infection confirmed by the high prevalence of HBsAg in the low income sub-Saharan Africa and East Asia and the heavy disease burden with its attendant consequences compared to the low and intermediate endemicity in other parts of the

world which are of middle and high incomes status gives an indication that poverty exacerbates the spread of the disease (Ott et al., 2012a).

Though some places had a slight increase in endemicity, there has being a general decrease in prevalence of HBsAg in some African countries such as The Gambia, Senegal, and Ivory Coast (Viviani et al, 1999; Coursaget et al 1994; Magoni et al 2009) and also in some Asian countries such as China and Taiwan (Lu et al., 2006, Liang et al., 2009). This is due to the mass infant and childhood immunization and improved methods of blood screening, safe injection practices especially across Africa and other countries around the world. The burden of hepatitis B in sub-Saharan Africa remains high at between 7% - 26% (Andre, 2000) as evidenced by the high mortality due to primary liver cancer in the region particularly among men (Ferlay et al., 2010, Di Bisceglie, 2009).

The vaccination program has its short falls as in a study; anti-HBc and anti-HBs were detected in 16.6% of the non-responsive individuals (about 2.3% of the whole study sample) indicating infection with the wild type virus. It is suggested that non response to the vaccine was probably due to infection before vaccination and females response to the vaccine was better than males (187.55IU/L verses 158.48IU/L) (Moradi et al., 2009). There is the need for universal antenatal screening for HBsAg, so positive mothers could be identified or better still a whole sale vaccination at birth should be encouraged for people living in high endemic areas like Ghana and other parts of Africa.

2.10 Significance of Perinatal Transmission

The source of most chronic infection in many countries is perinatal infection from mothers who are HBsAg positive with or without HBeAg positivity (ADHS report, 2012). Though small, transmission of hepatitis B in Ghana during the neonatal period of children is significant and may account for the high chronic endemicity of the viral infection in the country (Ghana EPI profile, 2002).The presence of HBeAg in the blood of mothers increases the risk of transmission from 5%-31% in HBsAg positive but HBeAg negative mothers (Alter et al., 1976, Beasley et al., 1977) to about 70%-90% in HBsAg positive and HBeAg positive mothers (Ott et al., 2012b, Hou et al., 2005). HBV DNA was found in children from HBsAg positive mothers even though the children are positive for anti-HBs. Thus occult HBV infection occur in vaccinated infants (Su et al., 2013). Despite increasing number of at risk children born in the United States, perinatal hepatitis B prevention programme is making progress in preventing a lot of perinatal infection (Smith et al., 2012) Seventeen babies out of 204 carrier mothers were infected through maternofeotal transmission when paired mother-cord blood or new born whole blood was tested for HBsAg and HBV DNA in the middle belt of Ghana. (Candotti et al., 2007). In the northern part of the country anti–HBs response was determined for infants to reach at least 87% (Hodgson et al., 2008). Another study in the section of the country also showed that vitamin A did not have any impact on the affinity of anti-HBs for the studied vaccine.

2.11 Genetics and the Hepatitis B Vaccine

The virus is classified into eight genotypes and all the genotypes vary by about 8% in the genome constitution. These genotypes are regrouped into 9 subtypes, 4 of which are described as principal subtypes: adw, adr, ayw, and ayr (Zuckerman, 2006). However, all combinations of the common determinants "a" and the mutually exclusive determinants are put into 9 subtypes- ayw1, ayw2, ayw3, ayw4, ayr, adw2,adw4, adrq1 and adrq2 (Mahoney, 1999). All subtypes share a common antigenic determinant "a" and contain other two pairs of mutually exclusive determinants (Zuckerman, 2006). The various hepatitis B vaccines available are either monovalent or combined. These vaccines could either be plasma derived or recombinant vaccine. Most of the hepatitis B vaccines available now are recombinant. These vaccines are produced by many companies the world over including Dynavax Technologies Ltd., Merck, GlaxoSmithKline, Sanofi, GenPhar, LG Life Sciences, Genexine and NanoBio Corp. Some of the vaccines produced by the pharmaceutical companies include Engerix-B, Recombivax HB, cormvax (Merck), Pediarix (GlaxoSmithKline), twinrix TM, Quinvaxem® inj., (Berna Biotech Korea Corporation). The Quinvaxem® inj. was in use at the time of sample collection but other brands of hepatitis B vaccines were used prior to this. The use of these vaccines has led to the mutation of the antigenic determinants, core and pre-s/s regions of the viral genes producing vaccine escape mutants (Su et al., 2013)

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study Site

The study site was the Princess Marie Louis Children's hospital (PML) Located on the Derbi Avenue, in the centre of Accra. It is a hospital devoted to the health needs of children providing services for children in the surroundings of the facility and those who are referred to the hospital from other health facilities within the region.

It consists of emergency and consulting rooms, theatres which were under construction at the time of this study, and admission wards. The catchment area of the hospital is Accra central which is densely populated and consist of the indigenes of the Ga state and several other people from all over the country.

3.2 Study Design

The study was a cross-sectional investigation of children aged 5–32 months most of whom reside in the catchment area of the health facility. Research participants were met on the day they attended the health facility; when consent was given by parents, recruitment was done, questionnaire was administered and blood sample taken at the laboratory the same day.

About one and half to two millilitres of venous blood sample was collected from children who met the selection criteria. Quantitative screening of the sera from the blood samples was done for anti-HBs using Antisurase B-96 ELISA (General Biologicals, Corp.,

Taiwan). Qualitative screening was done for anti-HBc-IgG using the immunoassays kits, Foresight® (Acon Laboratories, Inc., USA) and Anticorase B-96 ELISA (General Biologicals, Corp., Taiwan) to differentiate between vaccine responses and immune response due to exposure to the natural virus. To have a complete evaluation of entire immune response process, samples that tested negative for anti-HBs were screened for HBsAg using Surase B-96 ELISA (General Biologicals, Corp., Taiwan) and followed by Wondfo® rapid test (Guangzhou Wonfo Biotech Inc., China) to determine if vaccine failure was due to infection with HBV. Attempts were made to retrieve the hospital records (antenatal books) of the mothers to determine the HBsAg status of the mothers prior to parturition. Children welfare clinic attendance record cards were also reviewed to check vaccination status of children, vaccine batch numbers, date of birth and place of birth. The patients' folders of children were also reviewed to take the weight, diagnosis, blood transfusion record and immunological status of the children. Consequences of natural HIV or HCV co-infection was not considered among the factors.

3.2.1 Study questionnaire

A questionnaire was administered to parents who consented to the research. These questions sought personal data of mothers and children including ethnicity, area of residence, health service availability for both antenatal and post natal care, exposure to risk factors such as blood transfusion, parent's occupation, mother's hepatitis B status prior to parturition of the child, immune status and related disease, immune suppressant drugs intake, vaccination status of children and mothers (Appendix 1). Inpatients, whose

blood samples were brought to the laboratory, were traced back to the wards and questionnaire administered at the ward granted parents consented to the process.

3.2.2 Sampling

Convenience sampling method was used to recruit 446 participants who visited the hospital mainly due to ill-health. Both out and inpatients who met the inclusion criteria and had a venous blood sample drawn either at the ward or laboratory for any test were included in the study provided parents or guardians gave consent.

3.2.3 Sample size determination

With unknown hepatitis B antibodies response rate of children in the country, a prevalence of 0.5 was assumed together with, a standard score (z) of 1.96 at 95% confidence level and 5% allowable error margin. Using the equation, n $=\frac{z^2p(1-p)}{e^2}$, where **n**- is the minimum sample size, **z**-the standard score, **p**-the hepatitis B vaccine antibody response rate in children and **e**-the allowable error margin, the minimum number of study participants that were to be enrolled for the study was 385. Ten per cent of the minimum sample size was added to 385 to make room for at most 10% withdrawal.

3.3 Criteria for Selection of Study Participants

Inclusion Criteria

- All children age 5 months to 32 months who have received the pentavalent vaccine from child welfare clinic services at least one month earlier to the day of recruitment and/or blood sample collection were included.

- Children age 5 months to 32 months who visited the hospital for other health reasons, medical examination, and reviews without obvious liver ailments or jaundice were also included.

Exclusion Criteria

- Children age 5 months to 32 months who were too ill to donate blood sample
- Children whose parents or guardians objected to the study
- Children with liver diseases
- Known HIV seropositive children

3.4 Specimen Collection and Storage

Children were uniquely identified by a number and questionnaire administered to collect biodata of the mother and child. About 1.5*m*L-2*m*L of venous blood (whole) was collected once into evacuated EDTA tubes (BD vacutainer® K2 EDTA, Becton Dickson pre-analytical system, Belgium) by a phlebotomist at the hospital and samples were then transported in a cold box with ice to the Clinical Virology Laboratory of the University of Ghana Medical School for preparation. At the laboratory, blood samples were centrifuged at 4,500 rpm for 3 minutes at room temperature using EBA 20 table-top centrifuge (Hetteich Zentrifugen, Tuttlingen, Germany). Plasma was separated into sterile eppendorf tubes and stored at -20°C for a short while pending HBV marker analysis.

3.5 Laboratory Analysis

Serum samples stored at -20°C and ELISA kits stored at 4^0C were removed and allowed to thaw at room temperature for at least 30 minutes. Commercial quantitative and

qualitative ELISA namely Anti-surase B-96, surase B-96 (General Biologicals, Corp. Taiwan), Foresight (Acon Laboratories, Inc., USA) and Wondfo rapid test (Guangzhou Wondfo Biotech Co., Ltd, China), were used to determine the presence of anti-HBs, HBsAg, and anti-HBc-IgG. Anti-Surase B-96 was used to test all samples for anti-HBs and all anti-HBs negative samples were tested for HBsAg using surase B-96. Wondfo® rapid test for surface antigen was also used as a follow up test for the HBsAg. Samples that were positive for anti-HBs were also tested for anti-HBc-IgG with the foresight® EIA. The positive samples were retested with Anticorase B-96 (figure 3-1).

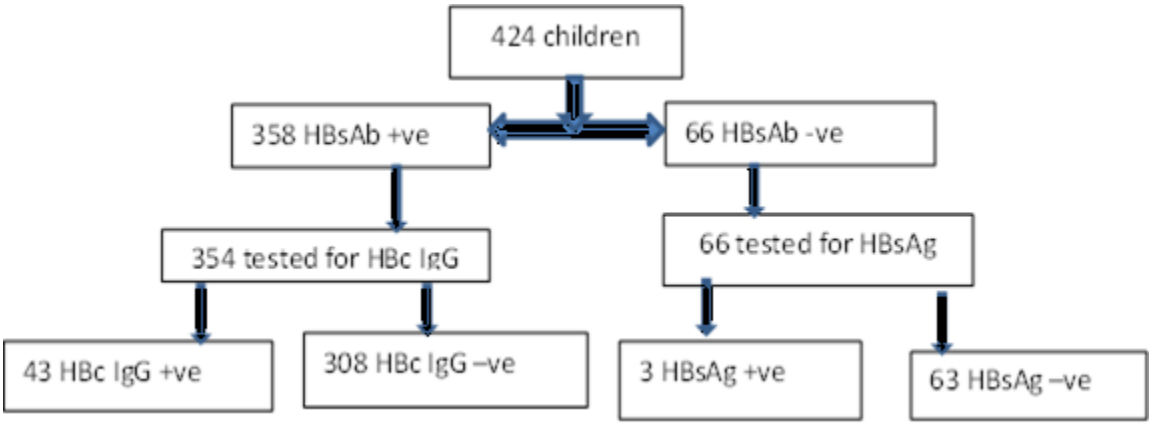

Figure 3-1: Summary of testing algorithm used for the study.

Legend: +ve, positive, -ve, negative.

3.5.1 Test for hepatitis B surface antibody (anti-HBs)

To determine the anti-HBs status of the children, each child's sample was tested in duplicates for anti-HBs as follows. The 1000 mIU/ml standard was diluted to 1/64 with normal human plasma that had been tested for anti-HBs, anti-HBc-IgM, anti-HBc-IgG, and HBsAg and found to be negative for all these markers to make 1/2,1/4,1/8, 1/16, 1/32, 1/64 dilutions. The first two wells of the microtitre plate were left as blank and 50

µl each of the negative control were placed in the next three wells. Fifty microliters each of the positive control was pipetted into the next two wells after the negative controls. Fifty microliters each of the dilutions and the 1000 mIU/ml, 100 mIU/ml, 10 mIU/ml, 0 mIU/ml standards were pipetted into duplicate wells of the microtitre plate.

Fifty microliters of each sample was pipetted into duplicates wells of the microtitre plate. Fifty microliters of the HBsAg·Peroxidase (conjugate) solution was added to all wells except the blank and incubated at 37 ± 1^{0}C for 1 hour. Wash buffer for washing the plate was prepared by diluting the concentrated buffer to 1:20 using distilled water. The plate was washed 5 times with the dilute/working solution, drained and blotted dry. Fifty microliters of TMB substrate A solution was pipetted into the washed microwells including the blank wells followed by 50 µl of TMB substrate B and \incubated at room temperature for 30 minutes. The substrates caused colour to develop in the positive wells while the negative wells remain colourless. The reaction was stopped by adding 100 µl of the stop solution (2N H_2SO_4) to each well and the optical density (OD) determined immediately using the spectrophotometer at 450nm. The cut-off value was calculated for each plate and the quality control and quality assurance parameters were checked and validated otherwise the samples were retested. Samples with optical densities below the cut-off value for each microtitre plate were considered non-reactive hence negative for anti-HBs. Those samples with optical densities above the cut-off value were considered reactive and therefore positive. Those samples within cut-off±10% range were retested and the results interpreted as above.

3.5.2 Test for hepatitis B core antibody immune globulin G (anti-HBc-IgG)

3.5.2.1 Using Foresight® Assay

The Foresight® ELISA assay was used to determine the anti-HBc status of the children who were positive for anti-HBs. Of the 358 children who were positive for anti-HBs, 354 were randomly selected and tested for anti-HBc-IgG. For each micro titre plate used, the first well was left as blank. With the exception of the blank wells, 100 µL of specimen diluent was added to each well of the microtitre plate including the positive and negative control wells.

Ten microliters each of the negative control was added to the next two wells after the blanks, and 10µL of the positive control was also added to the following two wells after the negative control wells. Ten microliters of each sample was pipetted into each of the rest of the wells of the microtitre plate. The plate was then sealed with the plate sealer mixed gently by swirling on the bench and incubated at 37 ± 2^0C for 30±2 minutes. The plate was then washed five times with working wash buffer (diluted from the concentrated buffer to 1:25), drained and blotted dry. One hundred microliters of conjugate was added to each microwell except the blank. The plate was sealed and incubated at 37 ± 2^0C for 30±2 minutes. The plate was then washed five times again with working wash buffer, drained and blotted dry. Fifty microlitres of substrate A solution was then added to each well followed by 50 µl of substrate B. The plate was gently swirled on the bench to mix and later sealed and incubated at 37 ± 2^0C for 10±1 minutes. Blue colour developed in wells that had the anti-HBc while the wells without the anti-HBc remained colourless. The reaction was then stopped by adding 50 µl of the stop

solution (0.5M H_2SO_4). The positive wells that initially developed blue colour changed to yellow while the negative wells (that were initially colourless) remained colourless. The ODs of the wells were determined immediately using the spectrophotometer at 450nm. The cut-off value of each plate was calculated following manufacturer instructions and the cut off value was used to determine if a sample was positive or negative. Those samples with OD below the cut-off value of the microtitre plate were considered non-reactive and therefore negative while those with optical densities above the cut-off value were regarded as initially reactive.

Those that were initially reactive were re-tested in duplicates and samples that produced at least one optical density value equal to or greater than the cut-off value were considered positive and were retested with another ELISA assay- Anticorase B-96 (General Biologicals corp. Taiwan).

3.5.2.2 Using Anticorase to test for anti-HBc-IgG

Some of the samples that were positive for the anti-HBc from the Foresight test were retested using Anticorase B-96 (TMB), General Biologicals Corp., Taiwan. In each microtitre plate for the test, 2 wells were reserve as blank. To the next three wells, 50 µL of the negative control was added while to the next two wells following the negative controls wells, 50 µL of positive control was added. To the remaining wells of the plate, 50 µL of each sample was added to each well. After addition of the samples, 50 µL of anti-HBc-peroxidase was added to each well except the blanks. The plate was gently tapped and then sealed tightly with the adhesive seal and incubated at 37 ± 1^0C for one hour. At the end of incubation, the seal was discarded and the plate washed six times with

washing solution prepared by diluting the concentrated washing solution D(20x) to 1:20, blotted and dried. Fifty microliters of the TMB substrate A was added into each well followed by 50 µL of TMB substrate B including the blanks and mix gently. The plate was covered with a black cover and incubated for 30 minutes at room temperature.

Blue colour developed in wells with samples that were negative for the anti-HBc. The reaction was stopped by adding 100 µL of 2N H_2SO_4 stop solution to all wells including the blanks, and this changed the colour of negative wells into yellow colour. The wells containing positive samples were colourless or light blue in colour. The absorbance of each well of the plate was read with the spectrophotometer at 450nm reading wave length with 620nm-690nm reference wavelength. The cut off value was calculated. All the quality controls and quality assurance conditions were met else the samples were retested. Samples with OD above the cut off value were regarded as negative while those with OD below the cut off are considered positive.

3.5.3 Test for hepatitis B surface antigen (HBsAg)

3.5.3.1 Surase B-96 test for hepatitis B surface antigen (HBsAg)

The hepatitis B surface antigen status of the children that were anti-HBs negative was determined by testing those samples for HBsAg using Surase B-96. The first well of the microtitre plate was reserved as blank. Fifty microliters each of negative control was placed in the next three wells while 50 µl each of the positive control was placed in the following two wells after the negative control wells. Fifty microliters of each sample was added to each of the rest of the wells of the microtitre plate.

Fifty microliters of horse radish peroxidase (anti-HBs.peroxidase made from guinea pig polyclonal antibodies and purified by affinity chromatography) solution (conjugate) was added to each of the wells except the blank. The plate was sealed tight with the adhesive slip and tapped gently to mix. This was then incubated at 37 ± 1^{0}C for 80 minutes and washed six times with Phosphate Buffered Saline (PBS) (diluted to 1:20 from the stock buffer) to remove unbound HBsAg. The plate was drained and blotted dry. Fifty microlitres of TMB substrate A was added to each well followed by 50 µl of TMB substrate B. The plate was then sealed tapped gently to mix and incubated at room temperature for 30 minutes. After addition of the substrate solutions, wells of the microtitre plate that had positive samples turned blue in colour while the wells with negative samples were colourless. This reaction was stopped by adding 100 µl of the stop solution (2N H_2SO_4) to each well and the OD of each well was determined immediately using the spectrophotometer at 450nm reading wavelength with 620nm-690nm reference wavelength.

The cut-off of the plate was calculated using the manufacturer's instructions. The samples with OD below the cut-off in each plate were considered non-reactive and therefore negative whiles those with OD above or equal to the cut-off value were considered initially reactive and the original samples were then retested in duplicates. When both ODs of the retested samples were less than the cut-off point they were considered negative. When at least one of the ODs of any of the retested samples was equal to or greater than the cut-off, then the sample was considered as repeated HBsAg positive and a follow up test to this was done with rapid detection kits.

3.5.3.2 Wondfo® Rapid detection test for the HBsAg

The rapid detection test kits used for the follow up test was Wondfo® (Guangzhou Wondfo Biotech Co., Ltd., China). To carry out the test, 100 µl of serum was added to the sample well of the test kit. It was then incubated for 15 minutes and the results read immediately after the 15 minutes. The positive samples had two rose-pink bands one at the control and the other at the test region while the negative samples had only one rose-pink band at the control. Two out of the three samples that were positive with the ELISA remained positive while one of the three that were positive with the ELISA turned out to be negative. All 66 samples tested for the HBsAg with the ELISA were tested for using this kit also.

All the test procedures for the various markers were in accordance with the manufacturer's instructions. For each plate ran, the validity of the test results were checked by making sure that all the quality control and quality assurance questions were true for the plate otherwise the test was repeated. The tests for the anti-HBs were performed in duplicates for reproducibility sake and for each ELISA microtitre plate, the cut-off point was calculated using the manufacturer instructions. The cut-off points were used to determine the positivity or negativity of the samples.

3.6 Quality Control

To ensure accurate results, internal and external quality control measures were taken. Whole blood samples were transported on ice in EDTA pre-coated tubes from the site to the laboratory. The internal quality control measures taken included wearing of appropriate attire (coat and latex gloves), cleaning of work bench before and after work

and immediately cleaning up spills when they occurred with 0.5% hypochlorite or 75% isopropanol as appropriate with the substance spilled.

Positive and negative controls contained in the kits acquired from General Biologicals Corp, Taiwan and foresight from Acon laboratories, USA- the producers of the ELISA kits were also tested alongside the samples. The concentration of the standards and their optical densities were used to plot a standard curve from which corresponding concentration of the optical densities of the samples were extrapolated.

Also, internal control samples with known antibody titres from the laboratory were ran alongside. Incubator's temperature was set at 37^0C whiles the wash buffer was diluted to appropriate concentration. Time for incubation and number of washings of microtitre plates were strictly adhered to and spectrophotometer reading done immediately after the stop solution was added as per manufacturer instructions. The plate layout was planned on a worksheet before a set of samples were tested. One plate was worked on at a time to avoid mix up. Cut-off values were calculated in accordance with the manufacturer instructions and quality control checks of the blanks, negative controls and positive controls values made to ensure the test was valid for each plate.

3.7 Statistical Analysis

Data entered into Microsoft Excel database was imported to IBM SPSS version 19 software (IBM Corporation, Somers, New York) and analysed. The variables chosen for analysis in the study were age, time between last dose and sample collection (period), same or different vaccines received, exclusive breast feeding, gender, diagnosis,

vaccination in scheduled times or out of schedule and immune response to vaccine through anti-HBs production.

The ages of children were calculated in months, and any number of days equal to or greater than two weeks was rounded up while any number of days less than two weeks was rounded down. Equally the period, which is time between the receipt of the last dose of the pentavalent vaccine and day of sample collection, was calculated in months and any number of days equal to or greater than two weeks was rounded up while any time less than two weeks was rounded down.

Diseases diagnosed by physicians on day of sample collection were categorized into those affecting the vascular system, those affecting the respiratory tract/system, those affecting the gastrointestinal tract, urinary tract infections, middle ear infection and others (diseases that could not put into any of the above mentioned broad categories). The mean hepatitis B antibody titre, age and period was determined, measure of central tendencies were calculated. Spearman correlation test was performed on all the variables to find out if there was any significant relationship between any two of these variables. Further statistical test was performed to determine the strength of the relationship between any two variable that showed any significant relationship from the spearman correlation test. Statistically significant differences between means of quantitative data were tested using Students' t-test while association between the categorical variables was determined using

chi squared (χ^2) statistic. A p value ≤ 0.05 was considered significant for all statistical tests.

3.8 Ethical Issues

The proposal was submitted to the ethical and review committee of the University of Ghana Medical School which approved the study. All data was handled anonymously to ensure high confidentiality. Identification numbers were assigned to the study participants and their blood samples and it was these numbers that were entered into excel for data analysis. Principal investigator made sure that all data/information about the study participants and completed code list was kept under lock and key. A voluntary written informed consent was sought from the parents or lawful guardian before children are recruited in to the study (appendix III). A translated version of the consent was provided for parents/guardians who could not read nor write English language. The researcher was at hand to explain some terms of the study which needed clarification. The details of the study, its benefits, and potential risk were declared to parents/guardians. Once all questions had been asked and clarifications made, a signature or thumb print of the parent/guardian was taken on the consent form as a sign of agreement to participate in the study.

CHAPTER FOUR

4.0 RESULTS

4.1 Enrolment

A total of 446 children were recruited with consent from parents or guardians from March 2012 to July 2012. Twenty-two of the participants were later excluded from the study due to incomplete personal data, insufficient plasma or parents declining after consent had been given. Therefore, 424 children were finally tested for anti-HBs to determine their immunity to HBV infection.

4.2. Demographic and Clinical Characteristics

Of the total number of participants tested, 215 (50.71%) and 209 (49.29%) were males and females, respectively. The mean age of the study participants was 13.7 months within a range of 5-32 months. The median age of the children was 13.0 months. The highest frequency was seen among children who were 11 months old (34 children) while those 28 and 31 months old had the lowest frequency. A detailed profile of the ages of the study population has been shown in Figure 4-1.

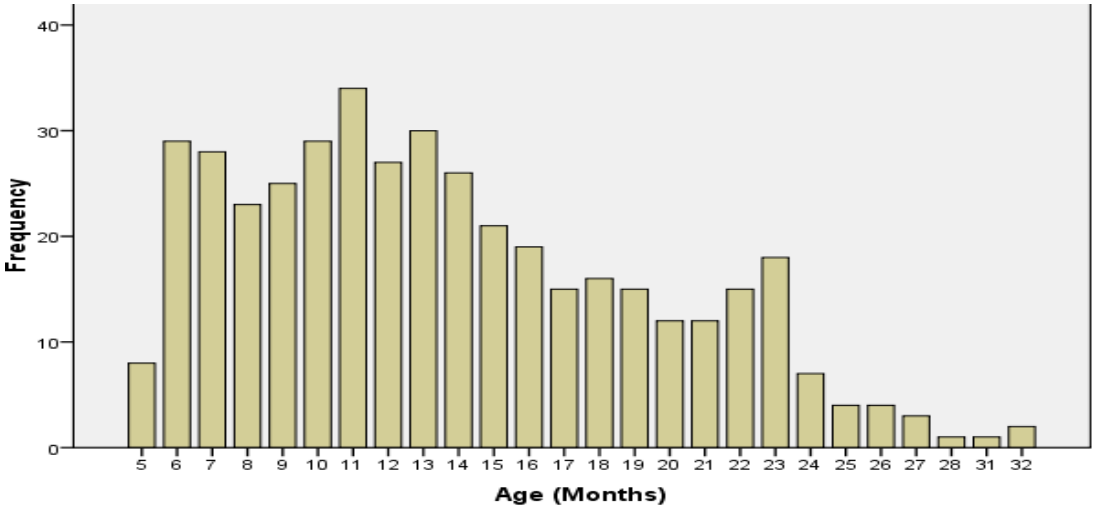

Figure 4-1: Age distribution of 424 children whose immunity against HBV was investigated

In order to rule out the effects of particular clinical conditions on vaccine responses, it was necessary to look at the distribution of the types of health conditions that brought the children to hospital. At the consulting room, the participants were diagnosed of diseases relating to the vascular system (such as malaria and sepsis), urinary tract infections, respiratory tract infections, anaemia, otitis media, gastrointestinal tract infections, and a few more that were collectively grouped into others. Those whose diagnosis was not known at the time of sample collection were considered one group. Majority, 268 (62.3%) of the children reported to hospital due to vascular related diseases while the least 8 (1.9%) was due to otitis media and also anaemia (Figure 4-2). The disease condition did not affect the detection of anti-HBs in the children (n=424; p=0.978).

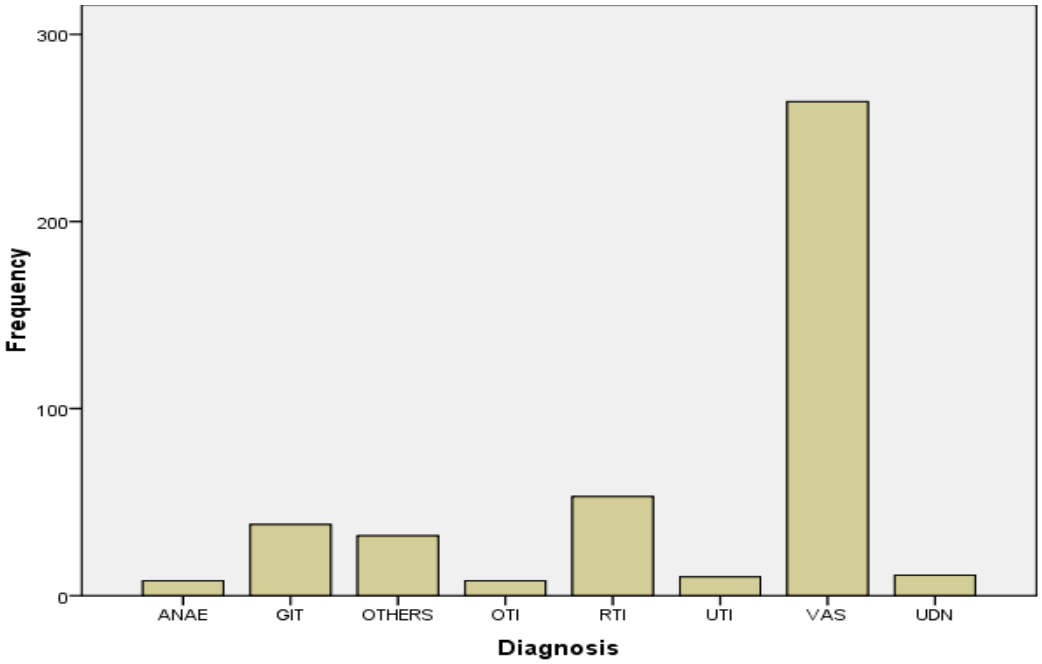

Figure 4-2: Disease conditions of 424 children whose immunity of HBV was determined

ANAE, anaemia; GIT, gastrointestinal tract infections; OTHERS, diseases with low frequencies which did not fall under the major groups identified; OTI, otitis media; RTI, respiratory tract infections; UTI, urinary tract infections; VAS, diseases associated with the vascular system; UDN, children for medical examination and without diagnosis.

4.3 Vaccination and Maternal Parameters

4.3.1 Vaccines

Not all the children took vaccines from the same manufacturer. Majority of the children, 268 (63.2%), received vaccines from the same manufacturer, while 155 (36.6%) and 1 (0.23%) had two and three doses from two and three manufacturers respectively.

4.3.2 Adherence to vaccination schedule

The timely administration of the vaccines to children at 6, 10 and 14 weeks was taken

into consideration during the analysis. Majority of the children, 333 (78.5%), took the vaccines on schedule times while 91 (21.5%) did not.

4.3.3 Time between last dose of vaccine and sample collection

The mean period (time between last dose of vaccine administration to children and day of blood sample collection) was 9.8 months. Majority of the children had their blood samples taken between 2 and 12 months after the 3rd dose of vaccine (Figure 4-3).

4.3.4 Exclusive breastfeeding

Two hundred and ninety-two (68.9%) of the children were reported as having been exclusively breastfed for the first 6 months of life while 132 (31.1%) had supplementary feeding in addition to the breastfeeding during the same period of life.

4.4 Hepatitis B viral markers

When the children were screened for different HBV markers, the prevalence of the HBV markers were as follow; 358 (84.4%) were positive for anti-HBs in the entire study population, 3 (0.7%) were positive for HBsAg, and 308 (n=354; 87.0%) were anti-HBc-IgG negative. Of the 358 who had anti-HBs, 340 (95.0%), 205 (57.3%), and 16 (4.5%) had titres ≥10 mIU/ml, ≥100 mIU/ml, and ≥1000 mIU/ml, respectively.

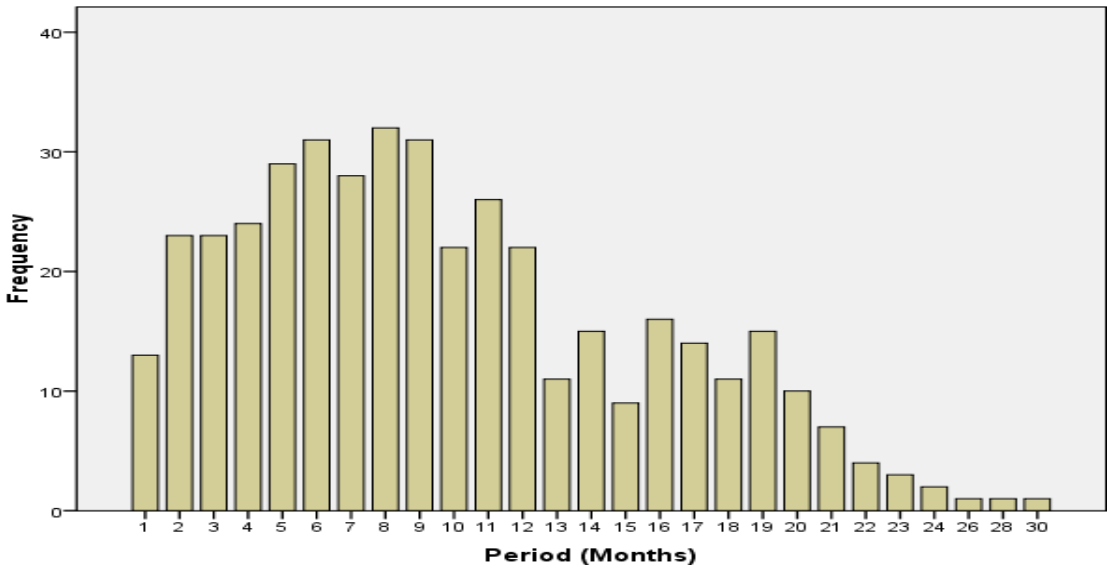

Figure 4-3: Time between last dose of vaccine and day of blood sample collection.

4.4.1 Hepatitis B surface antibody titres

Considering the classification used by Dahifar, 2004 and Hussain et al., 2005, almost half of the study population, 205 (48.3%), were hyper-responsive, 135 (31.8%) were hypo-responsive. Eleven children could not be classified because of discordance in the duplicate ODs of samples. Details of the results of various categories are shown in Table 4-1.

Table 4-1: Distribution of types of responders based on the comparison of anti-HBs titres of children.

Type of response*	No of participants (n=424)	Percentage (%)
Non-responsive	73	17.2
Hypo-responsive	135	31.8
Hyper-responsive	205	48.3
Indeterminate	11	2.6

***,** Non-responsive, <10 mIU/ml); hypo-responsive, between 10 and 100 mIU/ml; hyper-responsive, ≥100 m IU/ml; indeterminate, discordance in the duplicate sample runs for titres.

4.4.2 Hepatitis B surface antigen

When the 66anti-HBsnegative children were tested for HBsAg using Surase B-96 (General Biologicals corp., Taiwan) assay, 3 (4.5%) were HBsAg positive. Thus, in the total study population 0.7% of the participants were HBsAg positive. Two children were positive for HBsAg when both Surase (General Biologicals corp., Taiwan) and the Wondfo® rapid one step test (Guangzhou Wondfo Biotech Co. Ltd., China) were used, but the third child was not negative to the rapid test. However, the ODs of the surase B-96 for that third child was similar to the ODs of the other positive children.

One of the HBsAg positive children was 12 months old female. She was not exclusively breastfed and had received three doses of the pentavalent vaccine from two different manufacturers at the scheduled times at 6, 10 and 14 weeks of age. This child had never had any blood transfusion, and there was no record of any prophylaxis of HBV vaccine or HBIG at birth. The mother said she was tested for HBsAg during pregnancy and was negative but this could not be independently confirmed since she could not provide her antenatal record book for verification.

The second HBsAg positive child was 17 months old female and had taken three doses of the pentavalent vaccine from one manufacturer and at the required times. She had never been transfused with blood and was not exclusively breastfed. The mother tested positive for HBsAg during pregnancy, and this was confirmed from her antenatal record book. No record was available to ascertain if the child had received immunoglobulins or a vaccine at birth. The third HBsAg positive child was also female, 23 months old at the time of

sample collection and had received vaccines from a single manufacturer at the scheduled times. She never had any blood transfusion and was not exclusively breastfed. No records were available for the mothers HBsAg status and no records were available to show if prophylaxis was administered at birth.

4.4.3 The hepatitis B core antibody

Three hundred and fifty-four plasma samples were conveniently selected and screened for anti-HBc-IgG with Foresight® EIA (Acon laboratories Inc., USA). Majority of these children, 308 (87.5%) were negative while 43 (12.2%) were initially reactive or preliminary positive. However, only 6 (14.0%) of the preliminary positives had the ratio of OD/cut-off of the ELISA being >2. These 6 making 1.4% of the total study population were retested with another ELISA, AntiCorase B-96 assay (General Biologicals corp., Taiwan) and were positive. It is therefore unlikely that 37 out of the 43 initially reactive sera with low OD/cut-off ratio (1.01 to 1.44) were positive. In the absence of supplemental testing, these were considered as non-reactive. Of the 16 children who had anti-HBs above 1000 mIU/ml, all of them were negative for anti-HBc.

4.5 Correlates for Hepatitis B Surface Antibody Response

4.5.1 Pearson correlation between variables

The three outcomes investigated in this study were actual anti-HBs titres, anti-HBs titres that were adequate but with minimum protective levels against HBV infection (≥10 mIU/ml), and those with more than adequate protection (≥100 mIU/ml). In order to

determine the correlates for anti-HBs responses, Pearson's correlation was determined between all the variables and the three outcomes. In order to ascertain the robustness of the conclusions of this study and to exclude anti-HBs response due to natural infections, bivariate correlations and all analysis to determine the correlates for anti-HBs response, were done separately for the entire study population and for those who tested anti-HBc-IgG negative.

Variables that had a strong correlation with the three outcomes included the age of the children, the time between completion of three doses and enrolment for the study (period), and the different kinds of vaccines (from manufacturers) that were used. These have been shown in detail in Table 4-2. A Pearson's correlation was also performed among the variables other than anti-HBs to determine how they were associated. There were generally significant relationships between period, vaccine, timeliness and age. Details of these correlations are shown in Table 4-3. The other variables such as exclusive breast feeding, diagnosis and gender had no relationship with any of the other variables.

Table 4-2: Correlation between study variables and different anti-HBs outcomes

Variables	N=424∗		N=308ə		Actual anti-HBs titres	
	10 mIU/ml	100 mIU/ml	10 mIU/ml	100 mIU/ml	All (n=230)τ	HBcAb IgG neg (n=170)φ
Age	r=0.113; ***p=0.020***	r= 0.131;***p=0.007***	r=0.058; p=0.312	r=0.146; ***p=0.010***	r=-0.155; ***p=0.019***	r=-0.191; ***p=0.013***
Period	r=0.119; **p=0.014**	r=0.144; ***p=0.003***	r=0.063; p=0.271	r=0.162; **p=0.004**	r=-0.160; ***p=0.015***	r=-0.197; ***p=0.010***
Gender	r=0.066; p=0.174	r=0.110; ***p=0.023***	r=0.039; p=0.495	r=0.109; p=0.055	r=-0.024; p=0.721	r=-0.026; p=0.737
Timeliness	r=0.043; p=0.379	r= 0.011; p=0.816	r=-0.029; p=0.613	r=-0.061;p=0.289	r=-0.024; p=0.717	r=0.025; p=0.748
Feeding	r=-0.002; p=0.968	r=0.047; p=0.330	r=0.032; p=0.572	r=0.031; p=0591	r=-0.009; p=0.898	r=0.008; p=0.920
Vaccines	r=-0.072; p=0.136	r=0.135; ***p=0.005***	r=-0.111; p***=0.052***	r=-0.125;***p=0.028***	r=0.093,p=0.160	r=0.085; p=0.272

∗, All study participants; ə, study participants who had been tested for anti- HBc-IgG and were conclusively negative; τ, all study participants whose actual anti-HBs titres were known; φ, all study participants with actual anti-HBs titres and were anti-HBc-IgG negative; 10 mIU/ml and 100IU/ml, analysis where the minimum protection levels used in the analysis were equal to or above these values. Timeliness, whether the child received the vaccine on the schedule time of life –at 6, 10, 14 weeks of age; Feeding, whether the child was exclusively fed on only breast milk for the first six months of life); Vaccines, whether the child took all three vaccine doses from the same manufacturer or different manufacturers). Values in bold italics mean correlation is significant at the 0.05 level (2-tailed); r, Pearson's correlation coefficient; p, probability; neg, negative.

Table 4-3: Relationship between variables other than anti-HBs

Variable	Correlate with	r, p
Period (N=424)	Age	r = 0.98, p= 0.000**
	Vaccines	r= -0.255, p = 0.000**
Vaccines (N=424)	Age	r = -0.244, p = 0.000**
	Timeliness	r = 0.122, p = 0.012*
Period (N=308)	Vaccine	r = -0.283, p = 0.000**
	Age	r = -0.976, p = 0.000**
	Timeliness	r = -0.124, p =0.029*
Vaccine (N=308)	Age	r = -0.226, p = 0.000**
	Timeliness	r = 0.142, p = 0.013*
Period (N=230)	Age	r = 0.987, p = 0.000**
	Vaccines	r = -0.300, p = 0.000**
	Timeliness	r = -0.139, p =0.035*
Vaccine (N=230)	Age	r = -0.277, p = 0.000**
	Timeliness	r = 0.136, p = 0.04*

Period, time between the last vaccine dose and date of sample collection; vaccine, whether vaccine doses received by child were from the same manufacturer; timeliness, whether the vaccine doses were received on scheduled time of 6, 10, 14 weeks of child's age; r, Pearson-moment correlation coefficient; p, probability value; *, correlation significant at 0.05 level (2-tailed): **, correlation significant at 0.01 level (2-tailed);

4.5.2 Correlates for specific anti-HBs titres

When anti-HBs titre of 10 mIU/ml was used as the cut-off for protection, the age of the children and the period significantly influenced the outcome ($p<0.050$) (Table 4-4). Exclusive breastfeeding, gender, timeliness of vaccine administration, and the type of

vaccine combinations used did not determine if a child would have the minimum protective levels of anti-HBs or not (Table 4-4). These differences were not significant when the 308 children who had no anti-HBc-IgG were considered alone (Table 4-5).

Table 4-4: Comparison of characteristics between children with the minimum required anti-HBs protection (N=424)

VARIABLE	≥10 mIU/ml (n=340)	< 10 mIU/ml (n=84)	P-VALUE
Mean age (95% CI)	13.41 (-2.984 to -0.252)	15.02(-2.984 to-0.252)	0.020#
Mean period (95% CI)	9.46 (-3.113 to -0.345)	11.19(-3.113 to -0.345)	0.013#
Timeliness (yes) (%)	270 (79.4%)	63 (57%)	0.458*
Vaccine (type one) (%)	209 (61.5%)	59(70.2%)	0.136*
Gender (male) (%)	178 (52.4%)	37 (44.0%)	0.184*
Exclusive breastfeeding (yes) (%)	243 (71.5%)	58 (64.3%)	1.000*

Period, time in months between the last dose of vaccine taken by a participant and the date on which blood sample was taken; Timeliness, whether the child took the vaccine on scheduled times or not: Vaccine, whether the child took vaccine from same or different manufacturers; Exclusive breast feeding, whether or not mother fed child with only breast milk for the first six months of child's life: *,t- test of means (2-tailed significance); #, chi square test of association (2-tailed); ≥10 mIU/ml, refers to children whose samples had optical densities above or equal to that of the optical density of the 10 mIU/ml standard; < 10 mIU/ml, refers to children whose samples had ODs less than that of the optical density of the 10 mIU/ml standard.

Table 4-5: Comparison of characteristics between children with the minimum required anti-HBs for protection and were anti-HBc negative (N=308)

Variable	≥10 mIU/ml (n=290)	<10 mIU/ml(n=18)	p- value
Mean age (95%, CI)	13.44 (-3.966 to 1.189)	14.83 (-3.966 to 1.189)	0.274
Mean period (95%, CI)	9.46 (-4.203 to 1.121)	14.47 (-4.203 to 1.121)	0.441
Vaccine (one type)	175 (60.3)	15 (83.3)	0.052
Timeliness (yes, %)	227 (78.3)	15 (83.3)	0.612
Gender (%)	153 (52.8)	8 (44.4)	0.493
Exclusive breast feeding (%)	196 (67.6)	11 (61.1)	0.570

The relationships between the different variables and anti-HBs were more pronounced when the minimum cut-off for adequate protection was raised to 100 mIU/ml. The age of the children and the period significantly influenced the outcome strongly ($p<0.010$), and the vaccine combination was also essential in determining children that had 100 mIU/ml ($p=0.027$) (Table4-6). The strength of these relationships reduced slightly but still significant when only those without anti-HBc-IgG were used in the analysis (Table 4-7). The vaccine combination was also not essential in determining children that had 100 mIU/ml ($p=0.081$) (Table 4-7).

Table 4-6: Comparison of the characteristics of children with antibody titre below and above 100 mIU/ml (N=424)

Variable	≥100 mIU/ml (n=205)	< 100 mIU/ml (n=210)	p- value
Mean age (95% CI)	12.95 (-2.819 to -0.594)	14.43 (-2.819 to-0.594)	0.008
Mean period (95% CI)	8.92 (-2.585 to -0.389)	10.62 (-2.585 to -0.389)	0.003
Vaccine (one type) (%)	116 (56.6)	145 (69.0)	0.027
Timeliness (yes) (%)	163 (79.5)	162 (77.1)	0.628
Gender (%)	114 (55.6)	99 (47.1)	0.051
Exclusive breast feeding (%)	146 (71.2)	140(66.7)	0.599

Table 4-7: Comparison of characteristics of children with anti-HBs titres above and below 100 mIU/ml who are anti-HBc negative (N=308)

Variable	>100 mIU/ml (n=179)	<100 mIU/ml(n=122)	p- value
Mean age (95%, CI)	12.87 (-2.753 to -0.187)	14.34 (-2.753 to -0.187)	0.025
Mean period (95%, CI)	8.78 (-3.054 to -0.431)	10.52 (-3.054 to -0.431)	0.009
Vaccine (one type) (%)	101(56.4)	84 (68.9)	0.081
Timeliness (yes, %)	138 (77.7)	97 (79.5)	0.332
Gender (%)	100 (55.9)	60 (49.2)	0.066
Exclusive breast feeding (%)	123 (68.7)	79 (64.8)	0.750

The vaccine (number of manufacturers), the timeliness of vaccination, gender and exclusive breast feeding did not affect the intensity of anti-HBs titres in the 230 children with specific anti-HBs titres (p>0.050). A Wilcoxon's signed ranked test performed

showed that age and period influence the intensity of anti-HBs titre in children without anti-HBc (table 4-8). The vaccine (number of manufacturers), the timeliness of vaccination, gender and exclusive breast feeding did not affect the intensity of anti-HBs titres in the 170 out of the 230 children with specific anti-HBs titres but were negative for anti-HBc (p>0.050). A Wilcoxon's signed ranked test when performed showed that age and period significantly influence the intensity of anti-HBs titre in children without anti-HBc. Details of the results are shown in Tables 4-9

Table 4-8: Characteristics of variables in children with known anti-HBs titres (n=230)

Variable	Mean antibody titre (95% CI)	p-value
Mean age	10.728	0.000
Mean period	11.321	0.000
Timeliness (yes)	198.900 (-75.055 – 109.009)	0.717
Vaccine (type one) (%)	175.098 (-135.369 to 22.50)	0.160
Gender (male) (%)	202.381(-62.227 to 89.760)	0.721
Exclusive breastfeeding (yes) (%)	196.940 (-75.451 to 85.996)	0.898

Table 4-9: Characteristics of variables in children with known anti-HBs titres without anti-HBc (n=170)

Variable	Mean antibody titre (95% CI)	p-value
Mean age	10.745	0.000
Mean period	11.000	0.000
Timeliness (yes)	227.564 (-132.187 to 95.094)	0.748
Vaccine (one type) (%)	211.916 (-149.813 to 42.464)	0.272
Gender (male) (%)	239.604 (-77.027 to 108.733)	0.737
Exclusive breastfeeding (yes) (%)	229.755 (-102.560 to 92.558)	0.920

4.6 Univariate Analysis

Since the 230 children had actual anti-HBs concentrations, a Generalized Linear Model was used to determine the contributions of timeliness, age, vaccines and period in determining anti-HBs titres. The latter did not contribute significantly to the model hence had independent effects on the anti-HBs titres (Table 4-10).

Table 4-10: Tests of Between-Subjects Effects in children with anti-HBs titre above and below 100 mIU/ml

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	616964.733[a]	4	154241.183	1.840	.122
Intercept	662215.487	1	662215.487	7.901	.005
Period	64306.175	1	64306.175	.767	.382
Age	25564.751	1	25564.751	.305	.581
Timeliness	74672.665	1	74672.665	.891	.346
Vaccines	45948.155	1	45948.155	.548	.460
Error	1.886E7	225	83810.005		
Total	2.824E7	230			
Corrected Total	1.947E7	229			

a. R Squared = .032 (Adjusted R Squared = .014)

CHAPTER FIVE

5.0 DISCUSSION OF RESULTS

Hepatitis B is a vaccine preventable disease, and WHO in the mid-1990s decided that countries should integrate the hepatitis B vaccine into their EPI schedules especially those countries that are highly endemic. Ghana integrated the Hepatitis B vaccine into her EPI schedule in 2002 as one of the component of the pentavalent vaccine. There is sparse information on the effectiveness of the protection offered in preventing HBV infection in children in Ghana. Seroconversion to anti-HBs among the children to the vaccines administered was 84.4% which is consistent with other studies done in some countries (Azarka, 2004, Duval et al., 2005, Magoni et al., 2009). Even though 84.4% had anti-HBs with reference to the cut-off value from each microtitre plate, only 80.2% of the children had the protective anti-HBs titre of ≥10 mIU/ml. In a study to determine the anti-HBs rate of Iranian children to a Cuban hepatitis B vaccine 56.7%, 27.7% and 15.6% had ≥100 mIU/ml, between 10 and 100 mIU/ml and <10 mIU/ml respectively (Dahifar, 2004).The results of the current study is quite similar to this, but the protective rate of 84.4% in Dahifar's study was slightly higher than the 80.2% protective rate from the current study. It must be stated that the hypo-response rate (between 10 and 100 mIU/ml) of the current study is slightly higher while the hyper response rate (≥ 100 mIU/ml) is slightly lower compared to the study done by Dahifar in the year 2004. The genetic constitution of the individual has been found to be important in the immune response of the individual to infection and consequently the vaccines. The difference in the hypo-response rate could have been consequence of the genotype of the individuals involved with respect to their HLA types (Zuckerman, 2006). The effect of the type of vaccine and

the mode of vaccination including the vaccination schedule could not be ruled out since the studies were in different ethnic background (Zuckerman, 2006). The current study did not include these factors in the study and so no conclusion can be drawn with regard to these factors. However, since this study is in Africa and Dahifar's study in Asia, ethnic and genetic influence is bound to have an effect on differences of anti-HBs response to the vaccine. A study in this direction is compelling.

The persistence of protective anti-HBs titres strongly correlated with the strength of anti-HBs response after vaccination in this study. Fifthteen per cent of study participants who developed anti-HBs titre above a figure equivalent to >10 mIU/ml after vaccination lost all the anti-HBs while 27% of the same study population had their anti-HBs falling below 10 mIU/ml within the following five years (Hadler et al., 1986). From the study of Haddler, it follows that about 31.8% of the current study participants will lose the protective anti-HBs titres in about five years after the last vaccine dose. By extension, about 32% Ghanaian children will lose the protective anti-HBs levels five years after vaccination; hence, the need to check the antibody titre after the vaccination is completed. Possibly, extra dose of vaccine should be given to children irrespective of response rate and an appropriate study initiated to find the time at which maximum response to the booster dose is obtained. The non-response rate which stands at 15.6% can be compared to other studies in Africa and elsewhere (Magoni et al., 2009, Moradi et al., 2009, Azarkar, 2004, Esmaili and Seyedkolal, 2002). The non-response rate in the current study is slightly higher than that anticipated in hepatitis B vaccine immunization process where in a normal healthy human population, 5% - 10% non-response is anticipated (http://www.cdc.gov/vaccines/pubs/pinkbook/hepb.html). It must be mentioned that the

site for sample collection is a centre for malnourished children. Since nutritional level of the individual influences the immune status and consequently immune response (Rey-Cuille et al., 2012), high nonresponse rate was to be expected. The influence of mal-nutrition cannot be clearly stated since this was not one of the factors investigated. When nutrition status and country were simultaneously introduced into a multivariate model in Cameroun, only the country remained significantly associated with anti-HBs response (Re-Cuille et al, 2012). The study in the southern sector of the country will enable evaluation of the country with regards to the regions and zones of the country. Ghana of resent year has been struggling with inadequate and shortage of electric power supply. The effect of this on cold chain supply has not been investigated and the contribution of that to vaccine nonresponse is not known.

The mean ant-HBs titre of 230 children with known anti-HBs titres was 195.198 mIU/ml. The mean anti-HBs titres of the males was slightly higher (202.381mIU/ml) compared to the mean anti-HBs titre of females (188.641 mIU/ml) which contrast other findings where the mean male seroconversion rate was lower than the mean female seroconversion rate (Zeeshan et al., 2007, Moradi et al., 2009). Though a number of studies have shown female seroconversion rate to be higher than male seroconversion rate, no reasons have yet been found as to why this difference in seroconversion is observed. There appear to be a controversy on the effect of gender on anti-HBs response. While the results of the current study and others suggest that more males respond better to the vaccine (El-Sayed et al., 2011), others have indicated otherwise (Moradi et al., 2009, Zeeshan et al., 2007, El-Ghandour et al., 1998).

The ages of those with adequate immune protection was significantly different from those that did not have anti-HBs when anti-HBs titre of 10 mIU/ml and 100 mIU/ml were considered as cut-offs (p=0.02 and p=0.008 respectively). Thus the older children form the majority of those who do not have protective antibody level (tables 4-4 and 4-6), and this is consistent with the study of Van Damme and Van Herck, 2007. Thus, as children age the antibody levels produced as a result of vaccination wanes, hence, the need for a booster to maintain protective antibody levels in this high endemic zone.

Many children did not receive the vaccines on scheduled time, and it was anticipated that this could affect the immune response. However, the results of the study show that there was no correlation between anti-HBs response and timely receipt of vaccine. Equally, there was correlation between anti-HBs response and those that did not receive the vaccine on scheduled times. Those children that received the vaccine on scheduled times had mean antibody titre of 213.637mIU/ml while those that received the vaccine out of scheduled time had mean antibody titre of 214.125 mIU/ml, and the difference is not statistically significant ($p > 0.05$). Thus, provided a child received all the three doses of vaccine, the child is more likely to be immunised even when the vaccine was received out of scheduled time. Hence, defaulting children should be encouraged to take the remaining doses. However, if there is long stretch of time between one dose and the other, development of anti-HBs will also delay and within this time period of the receipt of the vaccine and development of anti-HBs, the child can be infected in a high hepatitis B endemic country such as Ghana. There is therefore, the need to avoid this behaviour. Equally, there is the need to find appropriate duration between doses of vaccine that can

produce maximum antibody response instead of relying on the current schedule which is not based on any study done in the country.

Seventy nine per cent of the participants who took the vaccine on time developed protective antibody titres of $\geq$ 10 mIU/ml while 57% of the participants who do not have protective antibody titre (< 10 mIU/ml) also received the vaccine on time. However, the difference between those that received the vaccine timely and were protected and those that were not protected even though they received the vaccine on time is not significant ($p = 0.458$). It cannot therefore, be concluded from this study that the timely receipt of the vaccine had on influence on anti-HBs response to vaccine.

The prevalence of the anti-HBc (IgG) among those that were positive for anti-HBs was 1.4% which is quite similar to other studies. In some studies, 0.7%, 1.38% and 5.2% of the study participants tested positive for anti-HBc (Duval et al., 2005, Abushady et al., 2011, Magoni et al., 2009). Anti-HBc-IgG prevalence was found in a study to increase with age but the increase was not statistical significant (Magoni et al., 2009). Thus, as children grow older they are more likely to be exposed to the virus; hence, the need for maintenance of protective anti-HBs-IgG titre through boosters. The lack of anti-HBc-IgG in the vaccinated children in the current study is very high comparable to the other studies mentioned (Duval et al., 2005, Abushady et al., 2011, Magoni et al., 2009). In some studies that have determined the anti-HBc (IgG) responses in children, anti-HBc-IgG had lower prevalence at younger ages (Magoni et al., 2009, Ni et al., 2001). Other

studies that did not test for anti-HBs also had similar results (Azarkar, 2004). It is therefore not surprising that when the analysis of the current study was done with or without anti-HBc-IgG, similar trends were observed (Duval et al., 2005).

It must be stated that all the study participants that had anti-HBs titres well over 1000 mIU/ml were all negative for the anti-HBc and so there was no external influence due to wild type HBV to that high anti-HBs response. Therefore, more than 72% of the children were protected solely by the receipt of the vaccine.

Considering the high prevalence of HBV in Ghanaian population (Martinson et al., 1996, Martinson et al., 1998), the prevalence of HBsAg in this study was very low. However, it must be mentioned that this study was conducted in one vaccination centre in the country, it stands to reason that cumulatively there will be a significant number of children who have received the pentavalent vaccine (DPT-HepB-Hib) and yet who will be infected with HBV. These could be breakthrough infection, problems with vaccine or site of vaccination and there is the need to ascertain the cause of this situation.

Compared to other findings in vaccinated children in Egypt, Gambia and Ivory Coast which had HBsAg prevalence of 0.8%, 0.7%and 0.7% (Reda et al., 2003, Van der Sande et al., 2006 and Magoni et al., 2009 respectively), the 0.7% HBsAg prevalence in the current study is similar. It could be assumed that the low HBeAg prevalence in mothers in Ghana (Acquaye and Mingle, 1994), the effectiveness of the vaccine and vaccination process may be responsible for this relatively low prevalence of HBsAg in the current study population as well as others in the West African sub-region. However, there is

need for caution as increasing prevalence of the HBsAg has been observed among pregnant women in Accra, Ghana (Damale et al., 2005). Furthermore, a high maternofoetal mode of transmission seen in the middle belt of Ghana (Candotti et al., 2007) implies that, more children are exposed to HBV between birth and time of first dose of vaccine. The viral load of mothers contribute immensely to the transmission of HBV to their children. Therefore, it is more likely that the mothers of the children who were positive for HBsAg had high viral load. Viral load of mothers is also associated with occult HBV infection (Su et al., 2013). The seemingly low HBsAg prevalence in vaccinated children in this study may be deceptive as it is believed that some of the children who were anti-HBs positive would had occult HBV infection. The prevalence of HBsAg in groups of study participants namely 2 to <4 year olds, 4 to 13 year olds and adults were 0%, 2% and 6.66% respectively in a study among vaccinated Egyptians (Abushady et al., 2011). Compared to the current study, 0.7% HBsAg prevalence among children age 5 to 32 months is on the high side and this could have been influenced by the high endemicity of HBV in the country. Vaccine mutant HBV and vaccine failure cannot be ruled out as the cause of the infections observed which may be break-through infections as observed in other places where HBV vaccines have been used (Chang, 2010, Su et al., 2012).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

In conclusion, anti-HBs response to the vaccine was good but many of those that responded were between 10 – 100 mIU/ml. The age, period, and vaccine (number of manufacturers) correlated with the anti-HBs response particularly when the anti-HBs response cut-off was pegged at 100 mIU/ml. Despite the vaccination, a small proportion of the children were still infected with the HBV. Children who received pentavalent vaccine from one manufacturer (producer) were more likely to develop protective antibodies than those that received vaccine from different producers. Additionally, it has shown that the prevalence and infection rate of HBV among the vaccinated children though small was still significant since the study was from only one out of the several vaccination centres across the country.

6.2 Recommendations

Administration of the vaccine at birth will be appropriate to curb the incidence of perinatal, inutero transmission and infection before 6 weeks of age when children receive the pentavalent vaccine. There is also the need to establish a nation-wide monitoring process to determine herd immunity of children who have received the vaccine.

Vaccines from different manufacturers should not be mixed or interchanged during administration. There is the need for a national policy, compelling testing of mothers during pregnancy for HBsAg to identify HBsAg positive mothers and give their children immunoprophylaxis at birth. Testing for the anti-HBs titre after vaccination is complete

and children with low anti-HBs titre (below 100 mIU/mL) are identified and given extra vaccine doses. There is the need for a booster dose that could be given at the same time with the measles vaccine (at nine months) to ensure the maintenance of the anti-HBs at the protective levels.

6.3 Limitations of the study

- Sensitivity of respondents to some questions limited study in terms of effect of ethnicity on vaccine response.
- Many mothers did not have their antennal record books with them so their status with regard to HBsAg before parturition could not be determined.
- HBV DNA was not determined thus, it was impossible to determine occult infection from chronic infection.
- Genotyping of the HBsAg was not done due to inadequate resources. Therefore, it could not be determined whether the infections were breakthrough or vaccine escape mutants.
- Test for HBeAb and HBeAg was not done, so contribution of HBeAg in the transmission of HBV from mother to child could not be analysed.

6.4 The need for further research

There is the need for this study to be extended to cover larger parts of the country. This will enable the unearthing of more data with regards to anti-HBs response to the hepatitis B antigen in the pentavalent vaccine.

Genotyping of the HBV in the HBsAg positive samples is absolutely necessary. This will reveal the genotype/sequence of the virus thus throwing more light on their genotype and whether they are vaccine escape mutant or wild. It will also be necessary to evaluate children for HBV DNA using molecular methods like (quantitative PCR/Nested) because this will be very useful in determining chronic or occult infection. Full panel test of the markers of hepatitis B will also significantly increase the knowledge of the contribution of the various markers to response or nonresponse of children to the vaccine.

It is also worth knowing the factors/cause that influence the difference in anti-HBs responses in male and female

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APPENDIX I

QUESTIONNAIRE:

UNIVERSITY OF GHANA MEDICAL SCHOOL

DEPARTMENT OF MICROBIOLOGY

STUDY QUESTIONNAIRE

Name of ward:……

Date of birth … …….. Sex: Male ☐ Female ☐

1. In which town was your ward born……..

2. Which region is your home region?.....

3. Which ethnic group do you belong? …….

4. Did you test for hepatitis B during pregnancy? Yes ☐ No ☐

5. What was the result? Positive ☐ Negative ☐

6. If positive in (5) above, did you receive any treatment? Yes ☐ No ☐

7. If negative did you receive any vaccine? Yes ☐ No ☐

8. If yes in (7) above how many times 1 ☐ 2 ☐ 3 ☐

9. Did you send your ward for any postnatal clinic services? Yes ☐ No ☐

10. If yes in (9) above did your ward receive the hepatitis B vaccine doses? Yes ☐ No ☐

11. Did your ward receive all the three (3) hepatitis B vaccinations? Yes ☐ No ☐

12. If no in (11) above, how many times did your ward receive the vaccine? 1 ☐ 2 ☐ 3 ☐

13. Has your ward ever been tested for hepatitis B Yes ☐ No ☐

14. If yes in (13) above what is his/her status? Positive ☐ Negative ☐

15. Has your ward ever been diagnosed of hepatitis B before? Yes ☐ No ☐

16. If yes in (15) above did your ward receive any treatment? Yes ☐ No ☐

17. Has your ward ever receive hepatitis B immune globulin? Yes ☐ No ☐

18. Has your ward ever received blood through transfusion? Yes ☐ No ☐

APPENDIX II:

CONSENT:

UNIVERSITY OF GHANA MEDICAL SCHOOL

COLLEGE OF HEALTH SCIENCES

Department of Microbiology

P. O. BOX 4236

Korle-Bu, Accra, Ghana

Participant ID Number:

Participant Name:

Study Title: **Response of children to the hepatitis B vaccine in the pentavalent vaccine in Accra.**

Vaccine:

Dear parent,

INFORMED CONSENT FORM

I am seeking your consent to enrol you and your ward in a study in which I shall test your ward for the hepatitis B virus following the hepatitis B vaccination during the post natal clinic attendance and to review your antenatal record card to find out your hepatitis B status before you gave birth. The vaccination program was started in 2002 and nobody knows how the Ghanaian children are responding to the current vaccine that is being used .i.e. whether the vaccine is inducing protection or not.

Hepatitis B is a blood borne viral disease that causes inflammation and destruction of the liver. When children contract this disease at an early stage in life, many of them become chronic carriers and a good percentage suffer cirrhosis and liver cancer later in life that

leads to death. It is transmitted from infected mother to child during birth, unsafe injections, and contact with infected body fluids from other infected children. Its mode of spread is similar to HIV but it spread faster than HIV. By the WHO standards Ghana is hepatitis B endemic and efforts are being made to stop this deadly disease from spreading. I am conducting a research into how children respond to the current vaccine which is being administered at health facilities by ministry of health and to find out the influence of the vaccine to the spread of the hepatitis B virus in children. I also want to find out if children of women who are hepatitis B positive are treated as required and how their children are responding to the treatment.

Willingness to Participate

You are to understand that:

1) You and your ward taking part in the research is entirely voluntary and that you can decide that you are not taking part in the research and nobody will object to that. Your refusal to take part will not influence the care you and your ward will subsequently receive at the health facility.
2) After agreeing for your ward to take part in the study you can change your mind and withdraw him/her from the study at any time without anybody objecting. This decision will again not affect the care you and your ward receives subsequently at the facility.
3) In the process of the study you will be required to answer questions about you and your ward personal life style and health. I shall also review your medical records. $1\frac{1}{2}$ml (about one tea spoon full) of your ward's blood will also be drawn from

his/her forearm once to determine how well he/she is responding to the hepatitis B vaccine that he/she has received.

Confidentiality

4) The information I shall obtain from you or your ward will be kept in the investigators confidence. In the event of scientific reporting of the findings of this study you or your ward will not be mentioned or identified by name.

Risk to Participant

5) The risks I can foresee are that you may not be comfortable answering questions about yourself or your ward. For example you may be worried that the researcher may share information about you or your wards life style and health status with others. Be assured that all information obtained from you or your ward will be confidential. Also the study will involve you spending considerable time answering questions of researcher and having some blood sample taken from your ward. Every possible precaution will be taken to ensure you don't waste unnecessary time than you need to at the hospital in relation to the research. You may also be anxious about the drawing of blood from your ward in connection with the study. The amount of blood that will be taken from your ward will not be different from what is taken when you come to the health facility for medical check-up. Please further understand that such amount of blood taken from your ward is safe and will not harm your ward. In addition your ward may experience some temporary discomfort or bruising at the site of blood drawn. This is however not more than one is likely to experience when one comes to the hospital for

normal laboratory tests. In any case, the phlebotomist and laboratory technicians at the hospitals are well equipped to handle that and the bruise normally will go away after some days.

Benefits to Patients

6) The potential benefit for your ward taking part in this study will be that test for viral hepatitis B will be performed for him/her free of charge. This will enable your Doctor appropriately advise you with regards to your wards hepatitis B status. Also the knowledge obtained from this study may help health professionals understand better the treatment and management of hepatitis B in Ghana.
7) You will not receive any financial compensation for participating in this study.

Section A: to be completed by researcher:

I have fully explained to …… the nature and purpose of the above described study procedure and the risk that are involved in its performance. I have asked the participant if he/she has any questions and I have answered all his/her questions

Full name of Researcher:…… Signature ……Date……

Section B: to be completed by the study participant

I have been fully informed of the above described study with its possible benefits and risk my child and I face in a language that I fully understand. I have been made to understand that the study is entirely voluntary and that my ward can refuse to take part in it or withdraw from it at any time without any consequences to the subsequent treatment and care my ward may receive at the health facility. I have been given the opportunity to ask questions that I have in relation to the research. All my questions have been answered to my satisfaction.

I have also been made to understand that **Thomas Apiung** of the microbiology department of the University of Ghana Medical School, the principal investigator for the study or his supervisor will be available to answer any questions I may have. I may contact **Thomas on 0208288653**or his supervisor **Dr Kwamena Sagoe on 0277408528.**

My signature or thumb print below indicates that I am willing for my ward to participate in this study.

Name of parent……Signature……Date……

Section c: to be completed by a witness to the consenting process

Name of witness……

Signature/thumbprint…… Date……

APPENDIX III

Simplified procedure of Surase B-96 (TMB)

Add 50µl controls (3x NC, 2x PC) and add 50µl per Specimen into wells. Reserve 2 wells for blanks.

↓

Add 50µl of Anti-HBs Peroxidase Solution into each reaction well, except 2 blanks.

↓

Incubate the plate at 37 ±1°C for 80 minutes.

↓

Wash the plate.

(Choose one of the following two methods for colour development).

↓

Add 50µl of TMB Substrate Solution A to wells and then add 50µl of TMB Substrate Solution B. Mix well, gently.

↓

Incubate at RT for 30 minutes.

↓

Add 100µl of 2N H_2SO_4 into each well.

↓

Determine absorbance at 450/ 650nm.

APPENDIX IV

DILUTON OF 100 mIU/ML STANDARD

To 125µL of free human plasma in eppendorf tube 125µL of the 1000 mIU/ml standard was added. It was mixed well.

↓

125µL of the 500mIU/ml was taken into another eppendorf tube and 125µL of free human plasma added and mixed well to make 125 mIU/ml dilution.

↓

125µL of the 125mIU/ml dilution was taken in to another eppendorf tube and 125µL of free human plasma added. It was mixed well to make 62.5 mIU/ml dilution.

↓

125µL of the 62.5 mIU/ml was taken into eppendorf tube and 125µL free human plasma added and mixed well to make 31.25 mIU/ml concentration.

↓

125µL of the 31.25 mIU/ml standard was taken into another eppendorf tube and 125µL of free human plasma added and mixed well to make 15.625 mIU/ml.

APPENDIX V

Protocol for anti-HBs (Antisurase B-96 TMB)

Allow samples and kits to thaw at room temperature for 30–60 minutes

↓

Prepare a Working Wash Buffer (WWB) 1:20

↓

Leave well A1 and A2 blank

↓

Add 50µL each of control (2XNC, 2XPC) to microtitre wells

↓

Add 50µL of standards, plasma, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 dilution into two wells each.

↓

Add 50µL of specimen to duplicate wells for the rest of the well in the plate.

↓

Add 50µL of conjugate to each well except blanks

↓

Tab to mix for 30 seconds, seal with plate sealer and incubate at 37^0 for 1 hour

↓

Remove seal and wash with WWB six times (350 µL in each well), drain plates

↓

Add 50 µL of substrate A to each well followed by50µL of substrate B

↓

Mix gently, Seal plate with plate sealer and incubate at R.T for 30minutes

↓

After 30 minutes remove plate sealer and add 100µL of stop solution into each well

↓

Read absorbance at 450/650nm immediately.

APPENDIX VI

Protocol for anti-HBc-IgG (Foresight®)

Allow samples and kits to thaw at room temperature for 30 -60 minutes

Prepare a Working Wash Buffer (WWB) 1:25

↓

Leave two wells blank, Add 100 µL of specimen diluent to all wells except blank

↓

Add 10µl controls (2x NC, 2x PC) and add 10µl per Specimen into wells.

↓

From F1 and following, add 10 µL of each specimen to each well

↓

Tab to mix, seal with plate sealer and incubate at 37^0 for 30minutes

↓

Remove seal and wash with WWB five times (350 µL in each well)

↓

Add 100µL of conjugate to each well except blank, seal and incubate plate at 37^0C for 30minutes

↓

Remove seal and wash with WWB five times (350 µL in each well), drain plate

↓

Add 50 µL of substrate A to each well followed by50 µL of substrate B

↓

Tab to mix on bench, seal with plate sealer and incubate at 37^0 for 10minutes

↓

After 10 minutes remove plate sealer and add 50 µL of stop solution into each well

↓

Read absorbance at 450/630-690nm immediately

APPENDIX VII

Protocol for anti-HBc-IgG (Anticorase B-96)

Allow samples and kits to thaw at room temperature for 30–60 minutes

↓

Prepare a Working Wash Buffer (WWB) 1:20

↓

Leave two wells for blanks

↓

Add 50µL of controls (3×NC, 2×PC) and add 50µL of each sample into the rest of the wells

↓

Add 50µL of conjugate (Anti HBc.peroxidase solution) to each well except blanks

↓

Tab to mix for 30 seconds, seal with plate sealer and incubate at 37±1^{0}C for 1 hour

↓

Remove seal and wash with WWB six times (350 µL in each well), drain plates

↓

Add 50 µL of substrate A TMB solution to each well followed by50µL of substrate B TMB solution

↓

Mix gently, Seal plate with plate sealer and incubate at RT for 30minutes

↓

Remove plate sealer and add 100µL of stop solution into each well

↓

Read absorbance at 450 (630-0nm) immediately.

APPENDIX VIII

Wondfo® rapid detection for HBsAg

Allow samples and kits to thaw at room temperature for 30 -60 minutes

↓

80µl to 100µl of serum was put into test well and left on a flat work bench

↓

Results read after 15 minutes

APPENDIX IX

Dilution of standards

The 1000 mIU/ml standard was used to make serial dilutions as follows;

To 125µL of free human plasma in eppendorf tube, 125µL of the 1000 mIU/ml standard was added. It was mixed well to make the 500 mIU/ml concentration. One hundred and twenty five microliters of the 500mIU/ml was taken into another eppendorf tube and 125µL of free human plasma added and mixed well to make 250 mIU/ml dilution. From the 250mIU/ml dilution, 125µL was taken in to another eppendorf tube and 125µL of free human plasma added. It was mixed well to make 125 mIU/ml dilution. From the 125 mIU/ml dilution, 125µL was taken into another eppendorf tube and 125µL of free human plasma added and mixed well to make 62.5 mIU/ml concentration. From the 62.5 mIU/ml dilution 125µL was taken into another eppendorf tube and 125µL of free human plasma added and mixed well to make 31.25 mIU/ml dilution. From the 31.25 mIU/ml dilution 125µL was taken into another eppendorf tube and 125µL of free human plasma added and mixed well to make 15.625 mIU/ml dilution.

APPENDIX X

Description of Variables and Data Management

All demographic, clinical and laboratory data was double entered into Excel (Microsoft Corp., Seattle, USA) spread sheet, coded, captured and cleaned. Parameters that were sought included

- Timeliness- whether the participant received the vaccine on scheduled times (i.e. at the 6th, 10th and 14th weeks of their life).
- Period –the time between the last vaccine dose was administered to participant and date of recruitment/sample collection.
- Vaccines- vaccine received by participants (whether from one producer or different producers)
- Diagnosis- ailment diagnosed by physician at the hospital. These were put into major groups as: Anaemia (ANAE), otitis media (OTI), urinary tract infection (UTI), respiratory tract infection (RTI), gastro intestinal tract infection (GIT), diseases affecting the vascular system (VAS) and others that could not be put into any of these major groups were lump together as OTHERS. Some participants who came for review or medical check-up or children who diagnosis was not known at the time of sample collection were designated (UDN).
- Protected -seeks to find if a participants has enough antibodies to protect him/her from the disease. It was done by comparing the ODs of the 10 mIU/ml standard to the samples optical densities. Those with optical densities less than 10 mIU/ml standard were regarded as not protected. The ODs of the standards and the samples were converted into concentration and the concentration of the samples

compared to that of the standards. Those with concentrations less than 10 mIU/ml were regarded as not protected.

- Other parameters include age of participants, anti-HBc-IgG (antibody to core hepatitis B immune globulin G), HBsAg (surface antigen to hepatitis B virus), and gender of the participants.

Data obtained was kept under lock and key and was accessible to only the investigator and his supervisor. Data obtained was not and will not be used for any other purpose or shared with other investigators.

APPENDIX XI

PRIMARY STUDY OUTCOMES

1. Primary qualitative outcomes

The cut-off value for each plate was used to delineate the samples that were positive hence had antibodies (anti-HBs or anti-HBc) from those that were negative and so had no antibodies. Equally, the cut-off value for each plate was used to delineate those that were positive for the surface antigen (HBsAg) hence infected at the time of sample collection and those negative for the HBsAg. For the Surase B-96, Antisurase B-96 and Foresight® ELISA assay, samples with ODs ≥ the cut-off value were positive while those with ODs below the cut-off were negative. However, for the Anticorase assay ODs below the cut-off values were positive for anti-HBc-IgG and those with ODs above the cut-off value of the microtitre plate were negative. For the wondfo® rapid test the appearance of two bands (test and control) was designated positive and those with only one band (control) were negative while those test with one band at the test were invalid and were retested.

The mean optical density of the 10 mIU/ml standard in each plate was used to separate samples that had OD ≥ that of the mean OD of the 10 mIU/ml standard hence protected against developing the disease from those that had ODs less than that of the mean 10 mIU/ml standard hence not protected. Those samples which had their duplicate ODs discordant with the mean OD of the 10 mIU/ml standard were declared indeterminate.

The ODs of the samples were also compared with the mean optical density of the 100 mIU/ml standard in each plate. Those samples that had ODs ≥ the mean OD of the 100 mIU/ml standard (hyper-responsive) were separated from those with OD < that of the

mean OD of the 100 mIU/ml standard. Those samples with duplicate ODs discordant with the OD of the mean OD of the 100 mIU/ml standard were regarded indeterminate.

Finally, the ODs of the samples in each microtitre plate were also compared with the mean optical density of the 1000 mIU/ml standard in each plate. Those samples that had ODs ≥ the mean OD of the 1000 mIU/ml standard were separated from those with OD < that of mean OD of the

100 mIU/ml standard. Those samples with duplicate ODs discordant with the mean OD of the 1000 mIU/ml standard were taken as indeterminate. Table X-1 below indicates the cut-off values of the plates in the test for HBsAb.

Table X-1: Cut-off values of microtitre plates for HBsAb

PLATE NUMBER	**CUTOFF**	**NC_X**	**PC_X**	**NC_X - PC_X**
7/01/2013	0.094	0.069	1.709	1.615
9/01/2013	0.082	0.057	1.714	1.657
10/012013A	0.088	0.063	1.661	1.598
10/01/2013B	0.084	0.059	1.126	1.067
11/01/2013A	0.078	0.053	1.266	1.213
11/01/2013B	0.080	0.055	1.805	1.750
14/01/2013A	0.078	0.053	0.776	0.723
14/01/2013B	0.082	0.057	1.767	1.710
14/01/2013C	0.079	0.054	1.329	1.275
15/01/2013A	0091	0.066	1.468	1.402
15/01/2013B	0.079	0054	1.343	1.289
16/01/2013A	0.082	0.057	1.726	1.669
16/01/2013B	0.082	0.057	1.699	1.642

NCx = negative cut-off, PCx = positive cut-off

QUALITY ASURANCE AND QUALITY CONTROL

The test validation requirements as well as quality control steps were observed including;

NC_x must be ≤ 0.2 otherwise test is invalid,

PCx must be ≥ 0.5 otherwise the test is invalid,

PCx - NC_x must be ≥ 0.3 otherwise test is invalid, were all met

Table X-2: Cut-off values of microtitre plates in the test for HBc IgG

PLATE NUMBER	CUTOFF	NC_X
24/01/2013A	0.147	0.007
24/01/2013B	0.139	-0.011
30/01/2013	0.137	0.013
4/02/2013	0.151	0.001
5/02/2013	0.143	0.007

The cut-off value for the microtitre plate for HBcAb using sapmles that were initially positive for anti-HBc from the test with foresight EIA was 1.114. Thus, ODs below these cut-off values were considered positive.

Also, the cut-off values for the microtitre plate in the test for HBsAg was calculated and found to be 0.091. Any OD above this value was considered positive and any value below this was considered negative for HBsAg.

In the test for all the HBV markers, the validation and quality assurance requirement were met.

2. Primary quantitative outcomes

The Data Analysis and Management System for ELISA (ADAMSEL-v1.1) was used to convert the optical densities of the plasma samples into actual concentrations. The mean OD of the serum that was used for making the dilutions was subtracted from the optical densities of the standards (only wells containing diluted standards) and the mean OD of the zero (0) standard was added to the OD of the 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 dilutions of the 1000 mIU/ml standard. This offset the effect of the normal human serum that was

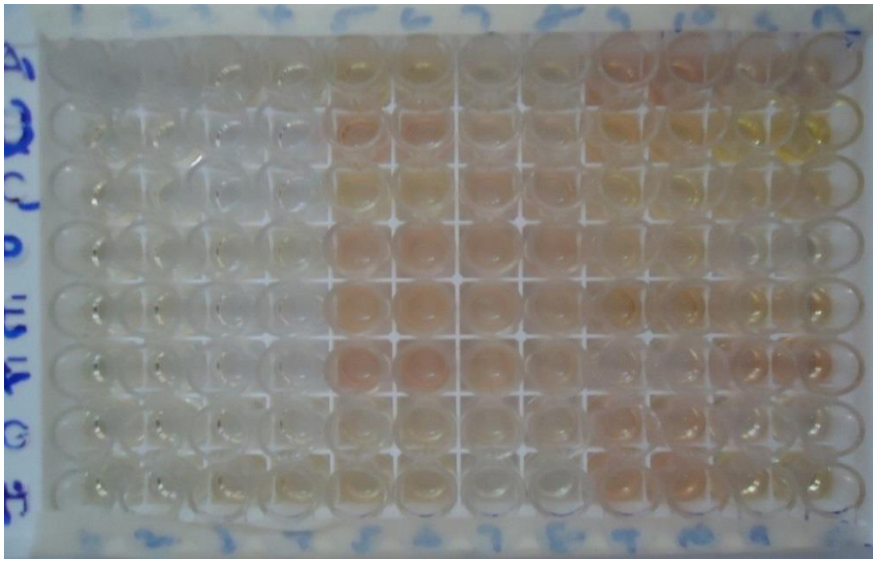
used to dilute the 1000 mIU/ml but also restored the effect of the zero standard which should have been used for the dilution but could not be used due to inadequate volume.

Optical densities of the dilutions, standards and samples of each plate were entered into ADAMSEL-v1.1. The software plotted a standard curve from the optical densities of the standards and dilution with the corresponding concentrations for each plate and from this curve the concentration of the various samples in each plate was estimated thus converting the optical densities of the samples into concentrations (titres). The average antibody concentration of the 10 mIU/ml standard in each plate was used to compare to the average antibody concentration of each sample in that plate and those with antibody concentration < the antibody concentration of the 10 mIU/ml standard were regarded as not having enough anti-HBs to protect them from the disease and those with average antibody concentration ≥ that of the average anti-HBs titre of the 10 mIU/ml standard were considered as being protected from the disease.

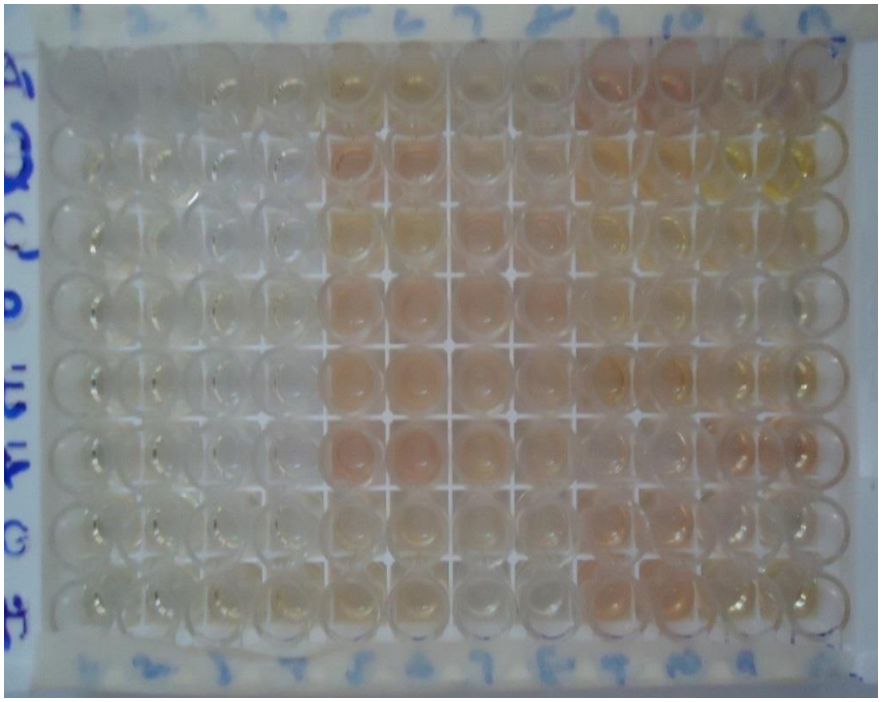
The average antibody concentration of the 100 mIU/ml standard in each plate was also used to compare to the average antibody concentration of each sample in the plate and those with antibody concentration ≥ 100 mIU/ml were identified as those with protection above 100 mIU/ml and were separated from those with average antibody concentration less than that of the 100 mIU/ml.

APPENDIX XII

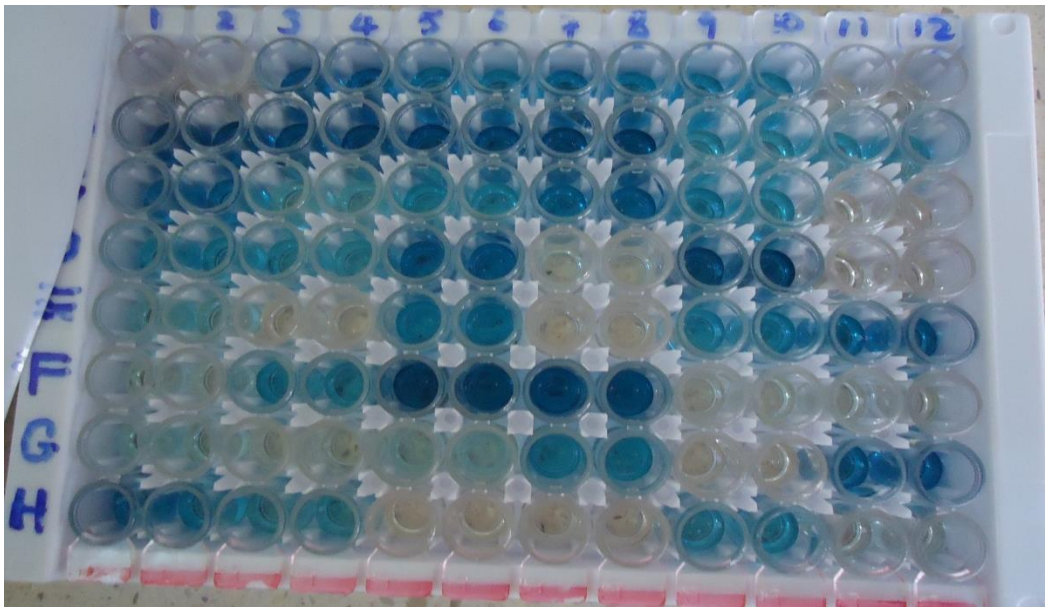
PICTURES OF TEST AT VARIOUS STAGES



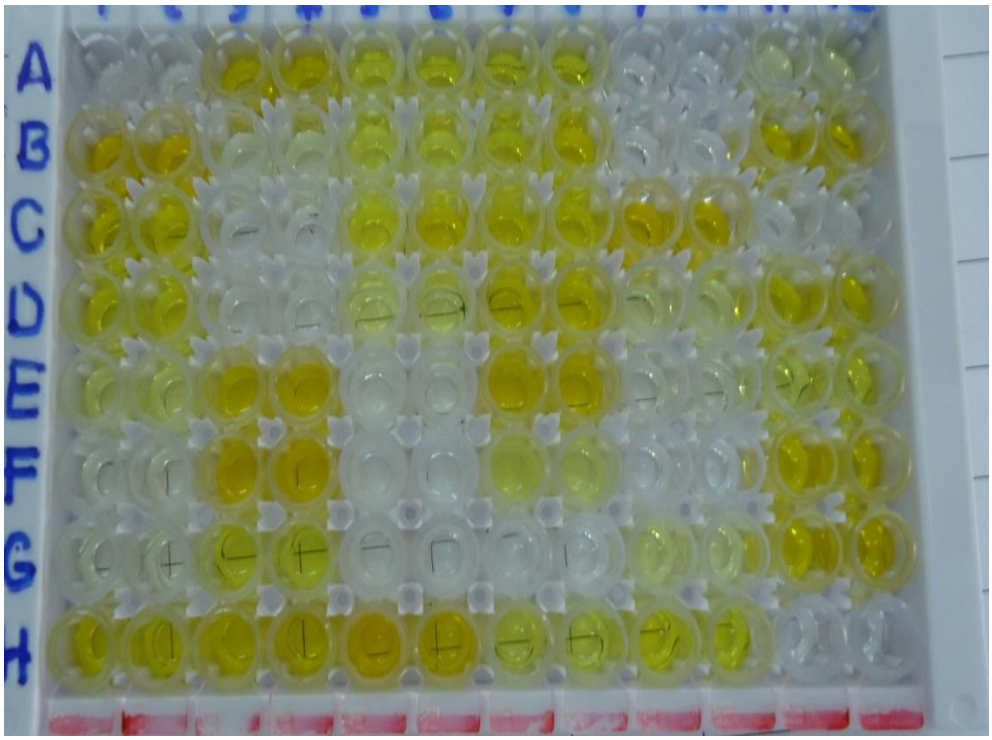
ELISA microtitre plate after controls, standards and samples have been added



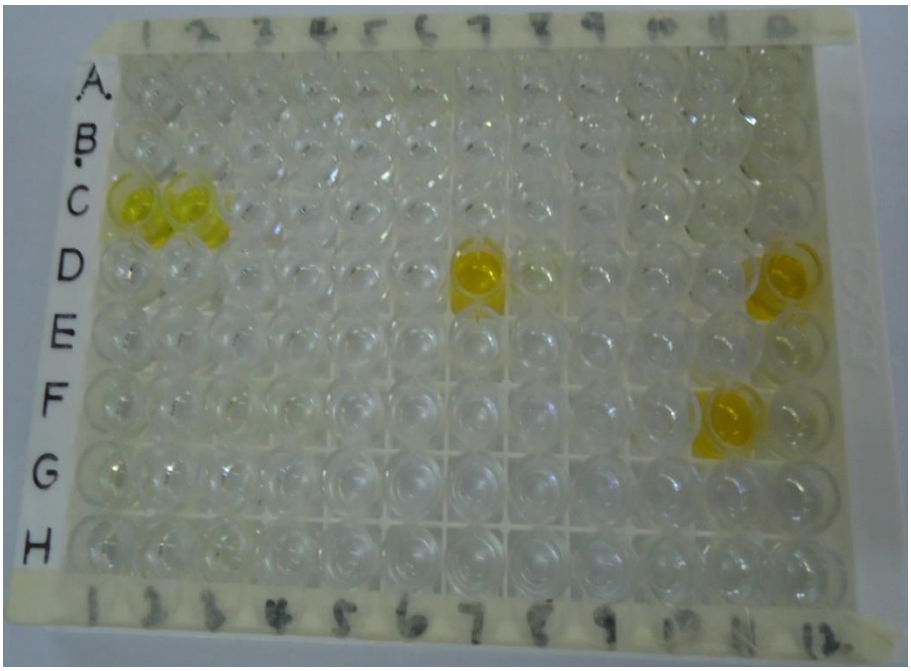
ELISA microtitre plate after anti-HBs.Peroxidase have been added



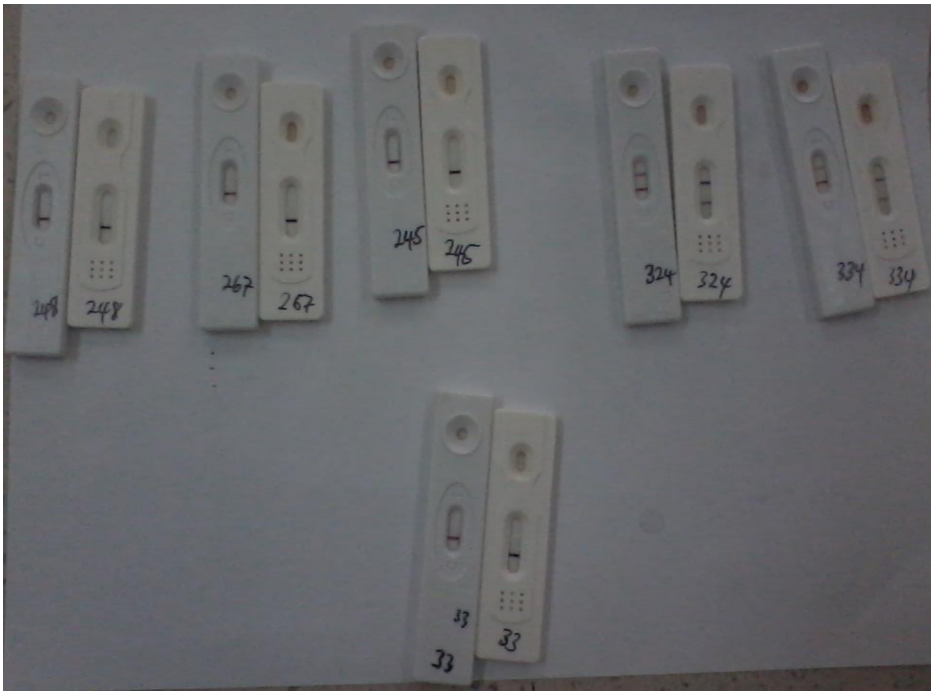
ELISA microtitre plate after substrate A and B TMB solutions have been added.



Test foranti-HBsafter stop solution has been added.



ELISA for HBsAg just after stop solution has been added



Rapid test for HBsAg using Wondfo® about 15minutes after adding sample to test well.