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AFLATOXIN LEVELS IN ADULTS WITH VIRAL HEPATITIS INFECTION IN THE EJURA-SEKYEDUMASE DISTRICT OF GHANA

BY

JUSTICE KUMI

THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF MPHIL CHEMICAL PATHOLOGY DEGREE

JULY, 2015

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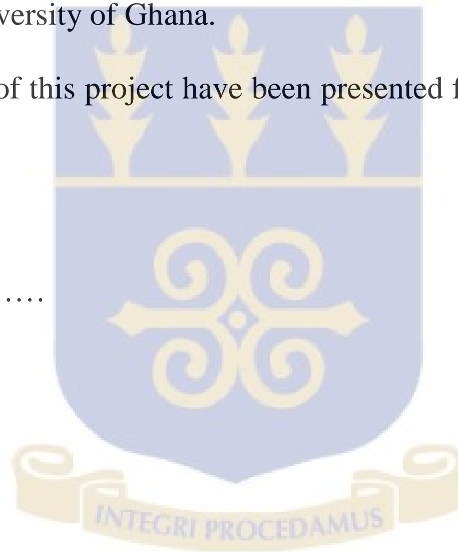
DECLARATION

I, **JUSTICE KUMI** hereby declare that with the exception of references to people's work, which have been duly acknowledged, this thesis is the outcome of my own research conducted at the Department of Chemical Pathology, School of Biomedical and Allied Health Sciences, College of Health Sciences, University of Ghana under the supervision of Dr. Seth Amanquah of the Department of Chemical Pathology, School of Biomedical and Allied Health Sciences of and Dr. Mark Ofosuhene, Department of Clinical Pathology of Noguchi Memorial Institute for Medical Research, College of Health Sciences, University of Ghana.

Neither all nor parts of this project have been presented for the award of another degree elsewhere.



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DEDICATION

This work is dedicated to my family

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My greatest gratitude goes to Dr. Seth Amanquah of the Chemical Pathology Department of the School of Biomedical and Allied Health Sciences (SBAHS), College of Health Science, University of Ghana for his uncountable contributions to this work.

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ABSTRACT

The consumption of foodstuffs contaminated with aflatoxins may lead to hepatotoxic effect particularly in coexistence with chronic viral hepatitis infection. In Sub-Saharan Africa, hepatocellular carcinoma is one of the most common malignancies. The aim of this study was to measure the level of exposure of aflatoxin and liver damage markers in adults with viral hepatitis in Ghana. The study was conducted in the Ejura-Sekyedumase district, Ghana. Five hundred adults were screened for viral hepatitis B and C. Spot urine and blood were collected and analysis of AFM_1 in the urine was measured using immunoaffinity column purification (AflaTest) followed by HPLC-flourescence detection at wavelength of 365 nm excitation and 425 nm emission . Viral hepatitis B and C and liver damage markers were measured from the blood serum samples using an automated colorimetric method. Out of total number of 500 samples screened 43(8.6%) tested positive for HBsAg only whiles 457 (91.4%) tested negative. Twelve samples tested positive for HCV. Fifty five samples tested negative for HBsAg and HCV for which were used as controls. There was significant difference in the AFM_1 levels between the HBsAg positive adults (5255.0±2757.9) and their controls (374.8± 227.7) (P=0.001). There was no significant difference in the ALT, AST, and albumin concentrations between the Hepatitis B surface antigen positive adults and the control group. Four adults (9.3%) tested positive for HBeAg with mean±SD concentration of $AFM_{1,}$ ALT and AST of 6536.7±5558.5 pg/dL, 58.0±12.4 U/L and 78.8±55.8 U/L respectively. Results from this study demonstrated significant levels of aflatoxin in HBsAg positive subjects compared to HBsAg negative subjects. The

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observations emphasize the need for aflatoxin exposure intervention strategies in high-risk countries; possibly targeted at postharvest food handling.

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LIST OF ABREVIATIONS

AFB_1……………………………………………………………..Aflatoxin B1

AFB_2……………………………………………………………..Aflatoxin B2

AFG_1…………………………………………………………….Aflatoxin G1

AFG_2…………………………………………………………….Aflatoxin G2

AFM_1...Aflatoxin metabolite

ALB…………………………………………………………………..Albumin

ALP……………………………………………………………..Alkaline phosphatase

ALT…………………………………………………………Alanine transaminase

AOAC……………………………………Association of official Analytical Chemist

AST……………………………………………………………...Aspartate transaminase

CDC……………………………………………………Centre for Disease Control

CMI…………………………………………………….....Cell-mediated immunity

CMOAT……………………………Canalicular multispecific organic anion transporter

CYP1A2……………………………………………………Cytochrome P450 1A2

FAO………………………………………………...Food and Agricultural Organisation

FDA………………………………………………..Food and Drug Administration

GAP……………………………………………………Good agricultural Practice

GGT…………………………………………………Gamma-glutamyl transferase

GST…………………………………………………………Glutathione S-transferase

HACCP…………………………………………Hazard Analysis Critical Control Point

HBsAg…………………………………………………Hepatitis B surface antigen

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HBsAb……………………………………………………..Hepatitis B surface antibody

HBeAg…………………………………………………Hepatitis B envelope antigen

HBeAb…………………………………………………...Hepatitis B envelope antibody

HBcAb……………………………………………………...Hepatitis B core antibody

HBV……………………………………………Hepatitis B viral deoxyribonucleic acid

HCC..Hepatocarcinoma

HCV…………………………………………………………………Hepatitis C virus

HSCAS…………………………………….Hydrated sodium calcium aluminosilicate

IARC…………………………………….International Agency For Research on Cancer

sIgA……………………………………………………..Secretory saliva immunoglobin A

MOFA…………………………………………...Ministry of Food and Agriculture

Ppb…………………………………………………………………Parts Per billion

Pg/dL……………………………………………………………...Picogram per decilitre

Pg/mL……………………………………………………………...Picogram per millitre

WHO……………………………………………………...World Health Organisation

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Aflatoxins are potent carcinogens that are produced as secondary metabolites by strains of the fungi *Aspergillus parasiticus* and *Aspergillus flavus* that grow and contaminate food crops such as groundnuts, maize and other oilseeds (Gourama and Bullerman, 1995). Aflatoxins exist in four major forms such as, B1, B2, G1 and G2 which occur together in different proportions in various foods (Gourama and Bullerman, 1995). Aflatoxin B1 is usually the predominant and most toxic form, and classified by the World Health Organisation as a class 1 carcinogen (Tseng, 1994).

A major metabolic product of aflatoxin B1 (AFB_1) is aflatoxin M1 (AFM_1) that is usually excreted in milk and urine of dairy cattle and other mammals (Gourama and Bullerman, 1995). Aflatoxin is one of the most important environmental toxins that contribute to the pathogenesis of hepatocarcinoma, especially in the regions where dietary foodstuffs (peanuts, corn, Brazil nuts, pistachios, spices and figs) are highly contaminated (Colombo, 1992; Qian *et al*., 1994). Aflatoxin B1 is metabolized by the liver through the cytochrome P450 enzyme system to the major carcinogenic metabolite AFB_1-8,9-epoxide. Aflatoxin B1-8,9-epoxide binds to cellular macromolecules including proteins and DNA to form adducts (Ross *et al*., 1992; Aguilar *et al*., 1993). Many studies have linked aflatoxin contamination of foods with acute illness resulting in death (CDC, 2004) and liver cancer (Bosch and Munoz, 1988; IARC, 1996 and Ozturk, 1991). Following ingestion of food contaminated with aflatoxin, it damages the

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liver and acts to synergize the development of hepatomas when other cancer causing agents (such as hepatitis B virus) are present (Wang *et al*., 1996). Approximately 4.5 billion of the world's populations are believed to be exposed to aflatoxins (Williams *et al.,* 2004).

Viral hepatitis is the inflammation of the liver, due to a viral infection that attacks the liver (National Library of Medicine, 2011). It may present in acute (recent infection, relatively rapid onset) or chronic forms (persistent). Chronic cases of viral hepatitis can lead to life-threatening liver cirrhosis, liver failure and liver cancer (National Library of Medicine, 2011). The most common causes of viral hepatitis are the five hepatotropic viruses, hepatitis A, hepatitis B, hepatitis C, hepatitis D, and hepatitis E. The global burden of viral hepatitis infection is high, with about 2 billion people exposed and 350 million individuals with chronic infection are at risk for developing liver disease (WHO, 2008). Hepatitis B viral (HBV) infection is estimated to cause 600,000 deaths each year, and about 25% of chronically infected adults later die from cirrhosis or liver cancer (WHO, 2008). Hepatitis B viral infection is estimated to cause 30% cirrhosis and 53% hepatocarcinoma (Perz, 2006). About 170 million people worldwide are chronically infected with hepatitis C viral (HCV) (IARC, 1994). Chronic infection of HBV and HCV is endemic in many developing countries of Sub-Saharan Africa, affecting over 20% of the population (Henry *et al*., 2002; McMahon, 2005). In Ghana prevalence of hepatitis B and hepatitis C viral infection have been reported as 10.53% and 5.63% respectively (Nkrumah *et al*., 2011).

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Hepatocellular carcinoma (HCC) is the fifth most common cancer and a major public health problem in the world (Parkin *et al*., 2005). In Ghana hepatocarcinoma mortality accounts for 21.15% of all cancer related deaths in adults (Wiredu and Armah, 2006). Hepatocarcinoma as a result of chronic aflatoxin exposure has been documented in persons with chronic HBV infection (Wild and Gong, 2010). It remains unclear whether aflatoxin exposure is an associated risk factor for advanced liver disease including liver cirrhosis in patients with chronic HCV infection (Chen *et al*., 1996; Sun *et al*., 2002). The risk of liver cancer in individuals exposed to chronic HBV infection and aflatoxin is greater than the risk in individuals exposed to aflatoxin only (Groopman *et al*., 2008). Studies have also shown that HBV positive persons have greater risk of aflatoxin exposure and that hepatitis B virus reduces the ability of the liver to detoxify aflatoxin in individuals (Allen *et al*., 1992). These two hepatocarcinoma (HCC) risk factors (aflatoxin and HBV) are prevalent in poor countries worldwide (Plymoth *et al*., 2009).

1.2 Problem Statement

Persons with viral hepatitis infection and exposure to aflatoxin stand the risk of developing liver diseases. Studies show that, the risk of aflatoxin to humans is usually the result of chronic dietary exposure which may be compounded by viral hepatitis infection (Wild *et al*., 1992). Aflatoxin has synergistic effect on hepatitis B virus-induced liver cancer (Kirk *et al*., 2006; Kuang *et al*., 2005). Approximately 250,000 deaths are caused by hepatocellular carcinomas in China and Sub-Saharan Africa annually and these are attributed to factors such as aflatoxin intake and incidence of viral hepatitis infection (Wild *et al*., 1992). Similar problem may exist in Ghana since

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large number of the population may take food contaminated with aflatoxin. However information relating to aflatoxin exposure and liver cancer is readily not available. Hepatocarcinoma mortality accounts for 21.15% of all cancer related deaths in Ghana (Wiredu and Armah, 2006). A study by Wild *et al.,* (1992) shows significant positive correlation between aflatoxin-albumin adduct and alanine aminotransferase (ALT). Anamika *et al*., (2013) also reported increased serum aspartate aminotransferase (AST) in mice induced with aflatoxin.

1.3 Justification

Studies in South-East Asia, Shanghai and Taiwan, have demonstrated an interaction between viral hepatitis infection and aflatoxins in determining the risk of developing liver diseases. The studies used liver damage markers and aflatoxin M1 to classify individuals for exposure status of aflatoxin. The study showed significant association with chronic infection in relation to hepatocarcinoma risk (Qian *et al*., 1994; Wang, 1996).

Aflatoxin exposure has been demonstrated in persons living in Ejura-Sekyedumase district in the Ashanti region of Ghana (Jolly *et al*., 2006; Kumi *et al.,* 2014). The findings of the study indicated the need for specifically targeted post-harvest food handling and preparation interventions designed to reduce aflatoxin exposure (Jolly *et al*., 2006). Previous work in the district showed high exposure rates of aflatoxins with people testing positive for the aflatoxin-albumin adduct and aflatoxin M1 (Wang *et al*., 2008). However, data on human aflatoxin exposure and the incidence of viral hepatitis is readily not available in Ghana. Knowledge of the local epidemiology of aflatoxin and

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viral hepatitis infection and their relation to the effect on liver damage markers are essential for predicting epidemics and planning for preventive measures. This will help to reduce level of the development of liver diseases. The need to initiate the estimation of aflatoxin and viral hepatitis exposure in a chronic aflatoxin exposed environment will provide a baseline data. This is essential requisite information needed in national planning and implementation of future strategies to reduce the incidence of liver diseases. The study therefore seeks to provide information on the incidence of aflatoxin and viral hepatitis exposure and its effect on liver damage markers.

1.4 Hypothesis

People with viral hepatitis infection have high levels of aflatoxin.

1.5 Aim

To measure the level of exposure of aflatoxin and liver damage markers in adults with viral hepatitis infection in Ejura-Sekyedumase district.

1.6 Specific Objectives

These were:

1. To determine urinary aflatoxin M1 concentrations in adults with viral hepatitis.
2. To determine the presence of hepatitis B surface antigen and their viral profile.
3. To determine the presence of hepatitis C viral antibodies.
4. To ascertain whether an association exist between urinary aflatoxin M1 and liver damage markers.

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CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Aflatoxins

The name aflatoxin was derived from a toxin producing fungus which caused a disease referred to as "Turkey X disease" in England in 1960 which resulted in the death of 100,000 young turkeys (Asao, 1963). The fungus was identified as *Aspergillus flavus* in 1961 and the toxin was named aflatoxin due to its origin (A. *flavis-Afla*). The Environmental Health Service on Line (EHSO) indicated further that, *Aspergillus flavus* is common and widespread in nature. The mould is found in the soil, decaying vegetation and grains undergoing microbial deterioration.

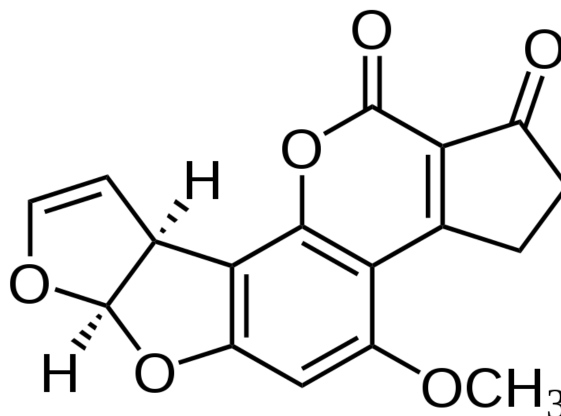

Figure 1. **Basic Structure of Aflatoxin**
(William *et al*., 2004)

Chemically, aflatoxins are difurocoumarolactones (difurocoumarin derivatives). Their structure consists of a bifuran ring fused to a coumarin nucleus with a pentenone ring in B and M aflatoxins (figure 1).

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2.1.1 The Genus *Aspergillus*

The genus *Aspergillus* belongs to the class deuteromycetes, phylum ascomycota in which a true sexual state is uncommon or unknown. Many of these fungi reproduce asexually by spores (conidia or oidia) or by budding. Their teleomorphs (life cycle) can be found in the ascomycetes. The fungi find many commodities as good substrate for growth, because of the large number of enzymes which they can use for their development (Hell, 1997). Pelczar and colleagues (1993) indicated that, the ascomycete produce sexual spores (ascospores) endogenously in a well-differentiated ascocarp (fruiting body) of an ascomycete fungus. Aflatoxins are products of many strains of *A. parasiticus* and *A. flavus.*

2.1.2 Chemical Nature of Aflatoxins

Aflatoxins are secondary metabolites, difuranocoumarin derivatives produced by a polyketide pathway. They are produced by species of *Aspergilli,* specifically *A. flavus* and *A. parasiticus*. The latter is a common contaminant in agriculture (Cullen & Newberne, 1993; Eaton *et al*., 1993; Bennett & Klich, 2003). These molds are ubiquitous in nature and grow on a variety of substrates, for example, dead organic matter, wood, clothes thereby producing aflatoxins. Aflatoxins are of great concern due to their biochemical and biological effects on living organisms (Ellis *et al*., 1991; Cullen & Newberne, 1993; Eaton *et al*., 1993; Shapira *et al*., 1996). Aflatoxins are a group of closely related compounds with small differences in chemical composition. Based on their fluorescence under UV light (blue or green) and relative

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chromatographic mobility during thin-layer chromatography. The four major aflatoxins are aflatoxin B1 (AFB_1), aflatoxin B2 (AFB_2), aflatoxin G1 (AFG_1), and aflatoxin G2 (AFG_2) (Ellis *et al*., 1991; Shapira *et al*., 1996).

2.1.3 Properties of Aflatoxins

Aflatoxin B1, B2, G1 and G2 were originally isolated by groups of investigators in England (Nesbitt *et al.,* 1962; Sargeant *et al.,* 1961). The fluorescence emission maximum for B1 and B2 has been reported to be 425 nm and that for G1 and G2 as 450 nm (Hell, 1997). The intensity of light emission, however, varies greatly among the four compounds, a property of significance in the estimation of concentrations of the compounds by fluorescence techniques. Some physical properties of the compounds are summarized in table 1.

Table 1 Properties of Aflatoxin

Chemical Formula	Molecular Weight	Melting Point (^oC)	Fluorescence
AFB_1 $C_{16}H_{12}O_6$	312	268-269*	425 nm
AFB_2 $C_{17}H_{14}O_6$	314	286-289*	425 nm
AFG_1 $C_{17}H_{12}O_7$	328	244-246*	450 nm
AFG_2 $C_{17}H_{14}O_7$	330	237-240*	450 nm

*Decomposes

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2.1.4 Conditions for Aflatoxin Growth

Production of aflatoxins is related to the highly variable relative humidity of the area, which influences moisture content of grains. Average relative humidity can be used to predict aflatoxin production (Dabbert and Oberheu, 2001; Williams *et al*., 2004). The toxins are produced as secondary metabolites by *A. flavus* and *A. parasiticus* fungi when the temperature is between 24°C and 35°C, humidity (<18%) and moisture content of > 7% (L'vova *et al*., 1984; Williams *et al*., 2004). Williams *et al*., (2004) concluded that, at latitudes between 40°N and 40°S of the equator in developing countries, contamination of stored, inadequately dried produce is responsible for fungal invasion and often begins before harvest and can be promoted by production and harvest conditions.

Aflatoxin is produced by fungal action during harvest, storage and food processing. It is considered by the United States Food and Drug Administration (FDA) to be an unavoidable contaminant of food such as maize (Plate 1), rice, cassava, nuts, chilies and spices (Williams *et al*., 2004).

Plate 1. Maize infested with *Aspergilus flavus* Source: Kumar *et al*., (2000)

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2.2 Toxicology of Aflatoxin

Poisoning that result from ingesting aflatoxins is known as aflatoxicosis. Two forms of aflatoxicosis have been identified: acute aflatoxicosis, which results in liver damage and subsequent illness and death. The second is chronic aflatoxicosis which is due to symptomatic exposure. A large number of studies have reported cases of aflatoxicosis in farm animals as well as wild life species in laboratory condition (Dabbert and Oberheu, 2001). After ingestion, the human gastrointestinal tract rapidly absorbs aflatoxins contaminated food and the circulatory system transports the aflatoxins to the liver. Aflatoxin is metabolized by cytochrome P450 group of enzymes in the liver, where it is converted to many metabolic products like aflatoxicol, aflatoxin Q1, aflatoxin P1, and aflatoxin M1, depending on the genetic predisposition of the species. Aflatoxin M1 is a toxic metabolite of aflatoxin B1. It is produced in the liver of animals and humans that have ingested aflatoxin contaminated commodities, primarily cereal grains. It is normally excreted in the urine and also secreted in milk or dairy product of cattle and other lactating mammals. The occurrence of aflatoxin M1 is transitory in nature, usually reaching a peak within 2 days after the ingestion of the contaminated commodity and disappearing within 4-5 days after the withdrawal of the contaminated source. Aflatoxin B1 is metabolized to a reactive aflatoxin-8, 9-epoxide. The amount of this metabolite interferes with the species susceptibility, as this can induce mutations by intercalating into DNA, by forming an adduct with guanine moiety in the DNA (Smela *et. al*., 2001) (figure 2).

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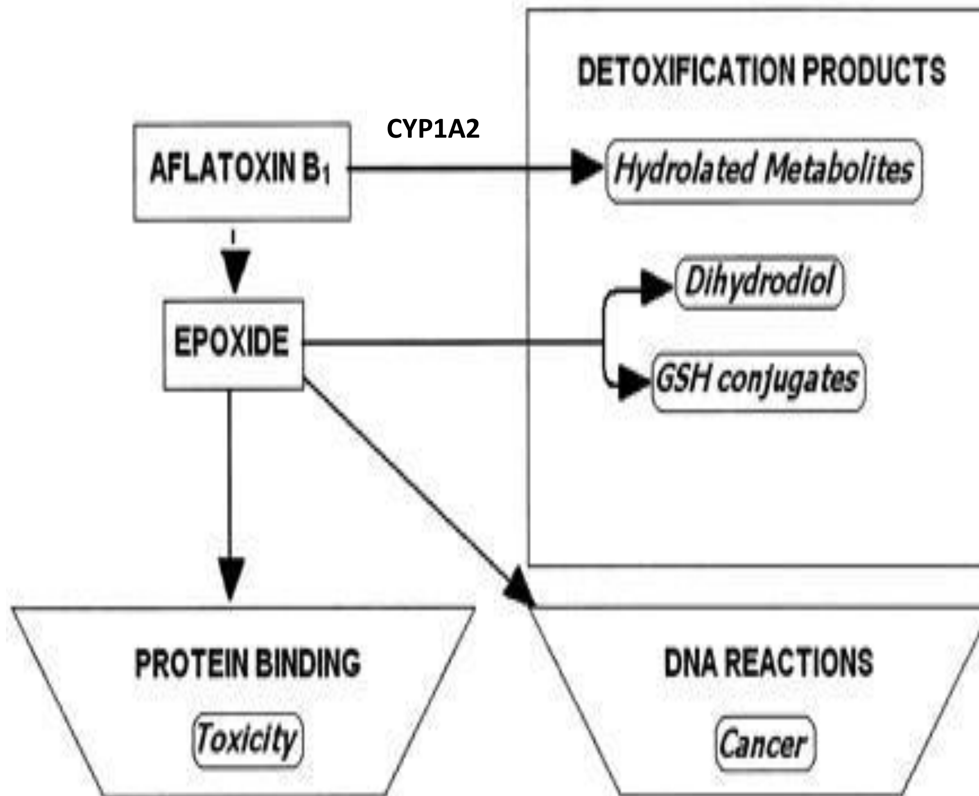

Figure 2. Pathways and consequences for aflatoxin in animal metabolism

(Williams *et al*., 2004)

Acute toxicity is caused when large doses of aflatoxin are ingested. The principal target organ for aflatoxins is the liver. After the invasion of aflatoxins into the liver, lipids infiltrate hepatocytes and leads to necrosis or liver cell death (Robens and Richard, 1992; Mclean, 1995). This is mainly because aflatoxin metabolites react negatively with different cell proteins, which leads to inhibition of carbohydrate, lipid metabolism and protein synthesis. In correlation with decrease in liver function, there is a derangement of the blood clotting mechanism, icterus (jaundice), and a decrease in essential serum proteins synthesized by the liver. General signs of aflatoxicosis are edema of the lower

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extremities, abdominal pain, and vomiting. The most severe case of acute poisoning of aflatoxin was reported in north-west India in 1974 where 25% of the exposed population died after ingestion maize contaminated with aflatoxin (Robens and Richard, 1992; Mclean, 1995).

Chronic toxicity is due to long term exposure of moderate to low aflatoxin concentration. The signs include decrease in growth rate and lowered milk or egg production in farm animals. Thus, a report in farm and laboratory animals chronically exposed to aflatoxins, interferes with protein metabolism and multiple micronutrients that are critical to health (Williams *et al.,* 2004). In addition, chronic exposure leads to a high risk of developing cancers, as aflatoxin metabolite (epoxide) can intercalate into DNA bases (Aguilar *et. al*., 1993). Moreover, food contaminated with aflatoxin may damage the liver and acts to synergize the development of hepatocarcinoma when other cancer causing agents such as hepatitis B virus are present (Wang *et al*., 1996).

2.3 Prevention and Control of Aflatoxicosis

To prevent aflatoxicosis it is necessary to explore public health interventions that promote effective production, storage and processing of homegrown and commercial grains. In addition, surveillance that monitors aflatoxin concentrations in food, use of specific processing and decontamination procedures may prevent widespread outbreaks of acute aflatoxicosis (Park, 2002). To ensure that foods consumed have the lowest aflatoxin concentration; this can be achieved for humans largely by regulations that require low concentrations of the toxin in exported food commodities (Williams *et al*., 2004). Other approaches to preventing aflatoxicosis involve chemoprotection and

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enterosorption (Galvano *et al*., 2001). Chemoprotection is based on manipulating the biochemical processing of aflatoxin to ensure detoxification rather than preventing biological exposure. Enterosorption is based on the approach of adding a binding agent to food to prevent the absorption of the toxin while the food is in the digestive tract; the combined toxin-sorbent is then excreted in the feces. This approach has been used extensively and with great success in the animal and human feeding industry (Rosa *et al*., 2001; Mitchell *et al*., 2014).

A key enterosorption agent, hydrated sodium calcium aluminosilicate (HSCAS) has been shown to effectively prevent aflatoxicosis in a number of laboratory and farm animals. The basic mechanism for HSCAS involves sequestration of aflatoxin in the gastrointestinal tract which results in elimination of aflatoxin in feaces (L'vova *et al*., 1984; Phillips *et al*., 1990). Bankole and Adebanjo (2003) have proposed other solutions to prevent aflatoxin production in Africa. These include education, drying, storage and smoking.

2.3.1 Education

The problem posed to the health and economy by aflatoxin is not widely known among the populace of most African countries. It is therefore necessary that the national agency in each country responsible for food safety, should embrace the task of creating awareness in the populace about the need to consume aflatoxin-free or good quality food. Private non-governmental organizations could also help to spread information on dangers of aflatoxin in both towns and villages. There should be regular education on

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radio and television on aflatoxin hazards and discussion on the issue should also feature regularly in daily newspapers and magazines.

Extension staff of the Ministry of Food and Agriculture (MOFA) in Ghana for example, could educate farmers or producers, on the need to adopt Good Agricultural Practices (GAP) to produce food free of aflatoxin. Hazard Analysis Critical Control Point (HACCP), a food safety control system based on a systematic identification and assessment of hazards in food and the identification of their control have been shown to be useful in food processing situations (Marriott, 1999). In an ideal HACCP-based system aflatoxins would be minimized at every phase of food processing and distribution.

2.3.2. Mechanical Drying

Among the recommendations for solving the aflatoxin problem, rapid drying of agricultural products to low moisture content is often emphasized, because all effects leading to aflatoxin contamination relate to non-maintenance of stored products at safe moisture content. Drying harvested maize to 15.5% moisture content or lower within 24 to 48 hours will reduce the risk of fungus growth and consequent aflatoxin production (Hamilton, 2000). Siriacha *et al.,* (1989) found that, if shelled grain was immediately sun-dried, contamination is reduced as compared with that of undried shelled maize. In Africa, most farmers sun-dry their harvests which often require longer durations for the product to attain moisture level of $< 7\%$ especially in times of cloudy weather. The grains are spread out on polyethylene sheets spread on the floor, and then

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stirring or turning is done manually till the product is dry. Due to the high rainfall amount at the time of harvest, farmers take some steps such as stacking the products to shield it from rain, drying grains over the fire (Amyot, 1983; Begun, 1991).

The effectiveness of drying was demonstrated in the report of Awuah and Ellis (2002) when groundnut kernels with 6.6% moisture were free of fungi regardless of the storage. However at 12% moisture, jute bags with *S. aromaticum* effectively suppressed the cross infection of healthy kernels. Since sun drying may be a difficult task due to the high rainfall at the time of harvest, work has been done on the design of solar and mechanical dryers for use by farmers in the tropics (Axtell and Bush, 1991). However, these dryers are not in use by farmers because large capital investment is involved.

2.3.3 The Use of Improved Storage Structures

Traditional storage structures used by farmers on the farm storage, include containers made of plant materials (wood, bamboo, thatch) or mud placed on raised platforms and covered with thatch or metal roofing sheet (Public Partnership Program, 2006). Essentially the storage structures are constructed to prevent insect and rodent attack and to prevent moisture from getting into the grains. The adoption of high yielding varieties which most often do not withstand long periods of storage by farmers has made the traditional storage systems to become inadequate. However, it has been very difficult to promote new storage systems such as the use of metal bins to small-scale farmers due to their high cost. Research is needed to develop and refine suitable storage systems that are not capital intensive.

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2.3.4. Smoking

Smoking is an efficient method of protecting maize against infestation by aflatoxin producing fungi. About 4 to 12% of farmers in the various ecological zones in Nigeria used smoke to preserve their grains, and this practice was found to lower aflatoxin levels in farmers' stores (Udoh *et al*., 2000). The efficiency of smoking to protect maize from fungal infestation was also confirmed in the survey conducted in Benin (Hell *et al*., 2000).

2.4 Aflatoxins in Stored Grains

A large number of researchers have demonstrated varying concentrations of different aflatoxins in a variety of foods stored in different conditions including rice bran, corn, maize and barley (Jayaraman and Kalyanasundaram, 1990; Wang *et al.,* 1995; Yoshizawa *et al*., 1996; Ali et *al*., 1998; Henry *et al*., 2002; Park, 2002). Jayaraman and Kalyanasundaram (1990) found that, the frequency of incidence as well as concentration AFB_1 increased with storage time in bran from untreated or raw rice and parboiled rice. It was discovered that, the rate of increase as well as overall concentration of AFB_1 were much higher in raw bran. Thus raw rice bran is unsuitable for prolonged storage. Park (2002) and L'vova *et al*., (1984) demonstrated formation of aflatoxin under the conditions of experimental storage of rice grain and reported that AFB_1 and AFG_1 are accumulated in the rice grain with initial moisture of greater than or equal to16%. Freitas and Brigido (1998) analysed peanuts and their products marketed in the region of Campinas, Brazil for AFB_1, AFB_2, AFG_1, and AFG_2 by thin-

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layer chromatography. The levels of AFB_1 reached the highest incidence compared with all the other aflatoxins.

In stored maize in Benin, Bouraima *et al*., (1993) found aflatoxin levels of 14 parts per billion (ppb) AFB_1 and 58 ppb for AFG_1. However, Setamou *et al*., (1997) reported that preharvest maize samples contaminated with aflatoxin was 42.5% and 30% in 1994 and 1995 respectively in the country. In addition, Hell *et al.* (2000) found that 9.9% to 32.2% of maize samples of different ecozones prepared for storage had aflatoxin levels more than 5 ppb in Benin. These levels increased to 15% and 32.2% after six months of storage.

In Nigeria, Udoh *et al*., (2000) reported 33% of maize samples from different ecological zones were contaminated with aflatoxin. However, in Ghana, maize samples from silos and warehouses contained aflatoxin levels in the range of 20 to 355 ppb; while fermented maize dough collected from major processing sites contained aflatoxin levels of 0.7 to 313 ppb (Kpodo, 1996).

Insects have also been reported as playing a role in the spread of *A. flavus* and increase in aflatoxin contamination (Setamou *et al*., 1998). The percentage of grains infected with *A. flavus* and samples contaminated with aflatoxin including the mean aflatoxin content of samples increased correspondingly with increased insect damage in preharvest maize in Benin (Setamou *et al*., 1998). Invariably, Hell *et al*., (2000) found

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out that, maize free of insect damage had no aflatoxin contamination, but maize with 70% of the cobs damaged by insects had 30.3% contaminated with aflatoxin.

2.5 Viral Hepatitis

Viral hepatitis is an inflammation of the liver due to a viral infection (National Library of Medicine, 2011). It may present in acute (recent infection, relatively rapid onset) or chronic forms. The most common causes of viral hepatitis are the five unrelated hepatotropic viruses (Hepatitis A, Hepatitis B, Hepatitis C, Hepatitis D, and Hepatitis E). Hepatitis A viral infection is caused by hepatitis A virus (HAV), a picornavirus transmitted by the fecal-oral route often associated with ingestion of contaminated food. It causes an acute form of hepatitis and does not have a chronic stage. The patient's immune system makes antibodies against HAV that confer immunity against future infection. Hepatitis A can be spread through consumption of raw sea food or drinking contaminated water. The time between the infection and the start of the illness averages 28 days (CDC, 2008).

Hepatitis B is an infectious disease caused by hepatitis B virus (HBV), a hepadnavirus that can cause both acute and chronic hepatitis. Chronic hepatitis develops in adults who are unable to eliminate the virus after an initial infection (WHO, 2014). Patients with chronic hepatitis B viral infection have antibodies against hepatitis B, but these antibodies are not enough to clear the infection of the affected liver cells. Identified methods of transmission include blood transfusion, sexual intercourse or through contact with blood or bodily fluids or via mother to child by breast feeding (WHO, 2014). Many people have no symptoms during the initial infection. Some develop a

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rapid onset of sickness with vomiting, yellow skin, feeling tired, dark urine and abdominal pain. It may take 30 to 180 days for symptoms to begin (WHO, 2014). Most of those with chronic disease have no symptoms; however, cirrhosis and liver cancer may eventually develop (Chang, 2007) and this can result in death (WHO, 2014). A vaccine is available that can prevent infection from hepatitis B for life.

Hepatitis C is caused by hepatitis C virus (HCV), an RNA virus, a member of the Flaviviridae family. Hepatitis C virus can be transmitted through sexual contact and can also cross the placenta. Hepatitis C usually leads to chronic hepatitis, culminating in cirrhosis in some people. It usually remains asymptomatic for decades. Hepatitis C viral levels can be reduced to undetectable levels by a combination of interferon and the antiviral drug ribavirin (CDC, 2010).

The Hepatitis D virus (HDV) can only propagate in the presence of the hepatitis B virus. On the other hand, hepatitis E virus (HEV) from the Hepeviridae family produces symptoms similar to hepatitis A, although it can take a fulminant course in some patients, particularly pregnant women; chronic infections may occur in immune-compromised patients.

2.5.1 Hepatitis B Viral Infection

2.5.1.1 Acute Hepatitis B Infection

The physical signs of typical acute hepatitis B infection may include variable degrees of jaundice, mild and slightly tender hepatomegaly and mild enlargement of spleen and lymph nodes. During acute hepatitis B infection, manifestations range from subclinical or anicteric hepatitis to icteric (jaundice) and, in some cases, fulminant hepatitis.

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Approximately 70% of patients with acute hepatitis B infection have subclinical or anicteric hepatitis. The average incubation period is 75 days (range 40–140 days). The onset of hepatitis B is typically insidious, with nonspecific symptoms of malaise, poor appetite and nausea. During the icteric phase, fatigue and anorexia usually worsen. Jaundice can last from a few days to several months, but usually 2–3 weeks. Itching and pale stools may occur. The convalescent phase begins with the resolution of jaundice. Laboratory testing during the acute phase of acute hepatitis B infection reveals elevated alanine and aspartate aminotransferase levels (ALT and AST). Values up to 1000–2000 IU/l are seen during the acute phase with ALT higher than AST. Serum alkaline phosphatase and lactic dehydrogenase are usually only mildly elevated. Bilirubin is variably increased, in both direct and indirect fractions. Serum bilirubin concentrations may be normal in patients with an icteric hepatitis. Serum albumin decreases especially in protracted severe hepatitis. During acute infection, hepatitis B viral surface antigen concentrations rise exponentially for weeks to months from undetectable to typical final concentrations of 10,000–100,000 ng/ml with 2–4 days of doubling time (Whalley *et al*., 2001). In acute hepatitis B, surface viral antigen decreases with an initial half-life of 8 days until it has disappeared completely from serum after weeks to months. In about 25% of cases of acute resolving hepatitis B, hepatitis B surface antigen disappears much faster, so that samples taken in the late acute phase may be hepatitis B surface antigen negative (Chulanov *et al*., 2000).

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2.5.1.2 Chronic Hepatitis B Infection

The natural course of chronic hepatitis B viral (HBV) infection consists of four phases. The immune tolerance phase which is characterized by the presence of hepatitis B envelope antigen (HBeAg), high hepatitis B viral (HBV) DNA levels, and persistently normal ALT levels, but no evidence of active liver disease.

The immune clearance phase is characterized by the presence of HBeAg and high/fluctuating HBV DNA and ALT levels. An outcome of the immune clearance phase is HBeAg seroconversion. Most patients then enter the inactive HBV carrier phase, which is characterized by the absence of HBeAg and the presence of anti-HBe, low or undetectable HBV DNA levels (<2000 IU/mL), normal ALT levels, and no/minimal inflammation on liver biopsy. The reactivation phase is characterized by the absence of HBeAg, intermittent/persistently increased ALT and HBV DNA levels, and inflammation on liver biopsy (figure 3). In persons who develop chronic hepatitis B infection, hepatitis B surface antigen is initially positive, accompanied by high levels of hepatitis B viral (HBV) DNA and may remain so for a few years to several decades (Lok *et al*., 1994; Chang *et al*., 1995).

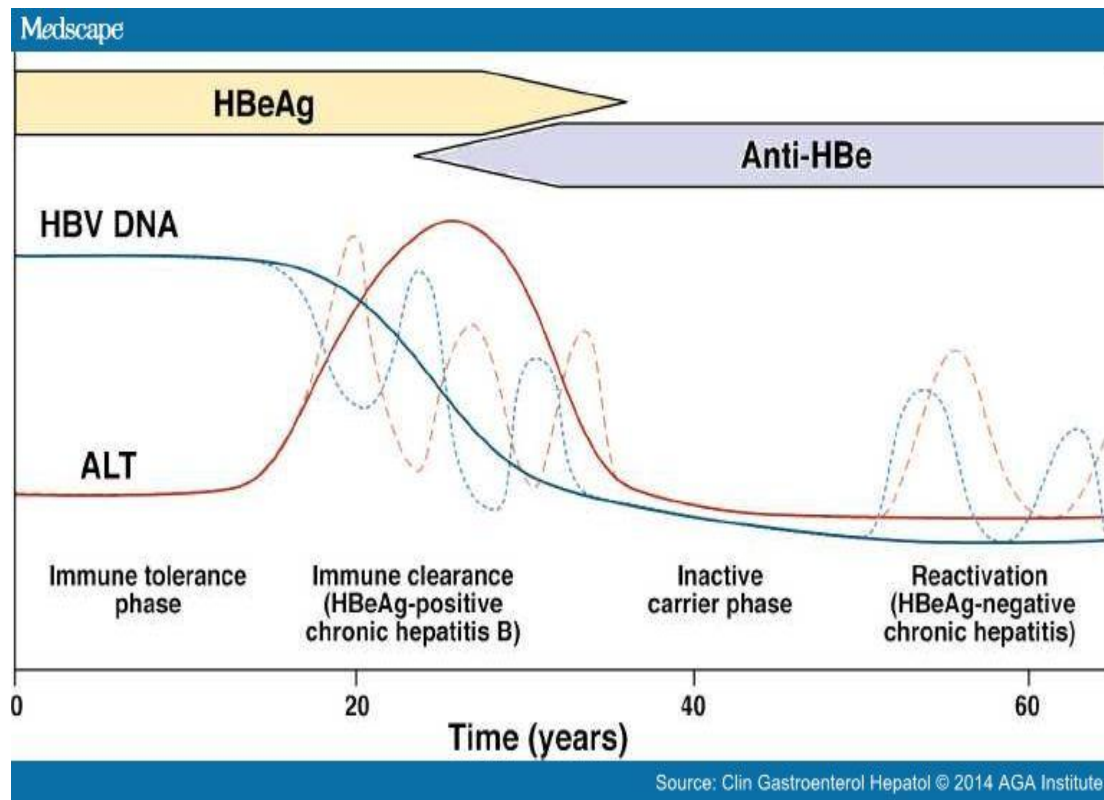

Fig. 3. Algorithm of natural history of chronic hepatitis B virus infection.

2.5.1.2.1 The Immune Tolerant Phase

Hepatitis B viral infected persons in the immune tolerant phase are HBeAg positive, have normal or low levels of aminotransferase (ALT and AST) up to 40 U/L levels, and elevated levels of hepatitis B viral DNA that are 20,000 IU/mL and commonly well above 1million IU/mL. The immune tolerant phase is thought to occur most frequently in persons who are infected via perinatal transmission from HBeAg positive mothers (Livingston *et al*., 2007). The immune tolerant phase is more frequent and more prolonged in subjects infected perinatally or in the first years of life. Because of

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high concentration of hepatitis B viral copies, is highly contagious. Hepatitis B envelope antigen may act as an immune tolerant protein that aids the virus in avoiding detection by the immune system. In immune competent persons, hepatitis B virus is not cytopathic and hepatocellular damage is induced by the host immune system's efforts to eliminate hepatitis B virus. The immune tolerant phase can last for a few years to more than 30 years (Hui *et al*., 2007). During this phase, there is either no or minimal liver inflammation or fibrosis. However, because the hepatitis B viral polymerase gene has reverse transcriptase properties, hepatitis B virus integrates randomly into the host's hepatocyte.

2.5.1.2.2 The Immune Active Phase

The immune active phase, also sometimes referred to as the "chronic hepatitis B phase" or the "immune clearance phase", is characterized by elevated levels of aminotransferases (ALT and AST) and an elevated hepatitis B viral DNA level above 2000 IU/ml. Active liver inflammation is usually present with or without liver fibrosis. Patients may be either hepatitis HBeAg positive or HBeAg negative (McMahon, 2005). Persons infected after birth who develop chronic hepatitis B viral infection may advance to the immune active phase shortly after the time of infection; whereas those infected via the perinatal route may transition into this phase several years after experiencing the immune tolerant phase of HBV. In this phase, the host's immune system recognizes HBV as being foreign and initiates an immune response that results in hepatocyte damage. In persons who are HBeAg-positive, HBV DNA levels may progressively fall,

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eventually result in seroconversion from HBeAg to hepatitis B envelope antibodies (anti HBe) (Lok *et al*., 1987; Liaw *et al*., 1983).

2.5.1.2.3 Inactive Hepatitis B Phase.

The inactive hepatitis B phase is characterized by the absence of HBeAg and the presence of anti-HBe, normal aminotransferase levels, HBV DNA<2000 IU/mL, and improvement in liver fibrosis and inflammation over time. Prospective studies conducted for up to 10 years of persons in the inactive hepatitis B phase have shown that in most of them, HBeAg remains negative, ALT levels remain normal, and HBV DNA levels remain <2000 IU/mL or even negative (de Franchis *et al.,* 1993; Zacharakis *et al*., 2005). Moreover, liver fibrosis is either absent or minimal in degree and shows no evidence of progression over time in those who remain in the inactive hepatitis B phase (Martinot-Peignoux *et al*., 2002 ; Zacharakis *et al*., 2005). However, a few clinic-based cross-sectional studies have demonstrated that minority of persons in the inactive HBV phase have had moderate or occasionally even severe fibrosis present on liver biopsy (Kumar *et al*., 2008).

2.5.1.2.4 The Reactivation Phase

In the reactivation chronic hepatitis B infection phase, patients are negative for HBeAg. Alanine aminotransferase (ALT) levels are normal and no or low levels of hepatitis B viral (HBV) DNA followed by acute episodes of marked ALT elevations and detection of hepatitis B viral (HBV) DNA (Hadziyannis , 2007). Hepatitis B virus can present in a fashion resembling acute infection (Davis and Hoofnagle, 1985; De Cock *et al*., 1986) and appears to have a high likelihood of resulting in cirrhosis (Fattovich *et al*., 1990;

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Hadziyannis, 2007). Spontaneous reactivation of chronic hepatitis B is often misdiagnosed (Paredes and Lewis, 2007) but this pattern of disease activity has been found to be quite responsive to antiviral therapy with nucleoside analogs which block the episodic flares of disease (Hadziyannis, 2007). Most instances of reactivation resolve spontaneously, but if immune suppression is continued, re-establishment of chronic hepatitis occurs which can lead to progressive liver injury and cirrhosis (Hadziyannis, 2007).

2.5 2. Markers of Viral Hepatitis B Infection

Hepatitis B surface antigen (HBsAg) is the earliest sign of an active hepatitis B infection. This antigen may be present before symptoms of HBV infection. If this antigen is present for more than 6 months, then a person will probably have a chronic (long-term) HBV infection. This means one can spread HBV to others throughout life (Hollinger and Liang, 2001).

Hepatitis B surface antibody (HBsAb) usually appears about 4 weeks after HBsAg disappears. The presence of this antibody means that the infection is at the end of its active stage and cannot pass the virus to others. This antibody also protects one from getting HBV again in the future. The test is done to determine the need for vaccination. The antibody will be present after receiving the HBV vaccine series, which gives protection (immunity) from the virus infection. Occasionally the test may show both HBsAb and HBsAg (Hollinger and Liang, 2001).

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Hepatitis B envelope antigen (HBeAg) is an HBV protein that is only present during an active HBV infection. This test determines how contagious the disease is to others. Testing for this antigen can also be used to monitor the effectiveness of treatment for HBV (Hollinger and Liang, 2001).

Hepatitis B envelope antibody (HBeAb) shows that the active stage of an acute HBV infection is almost over and the risk of disease being contagious is reduced (Hollinger and Liang, 2001).

Hepatitis B viral (HBV) DNA testing checks DNA from the hepatitis B virus. The HBV DNA tests measure how much genetic material is present. High levels of HBV DNA means that the virus is multiplying in the body and it can be very contagious. In chronic HBV infection, an elevated viral DNA level means there is an increased risk for liver damage. Testing for hepatitis B viral (HBV) DNA is used to check the effectiveness of treatment for long-term (chronic) HBV infection (Hollinger and Liang, 2001).

Hepatitis B core antibody (HBcAb) is an antibody to the hepatitis B core antigen that appears about 1 month after the start of an active HBV infection. It can be found in people who had an infection in the past and in those with long-term (chronic) HBV infection. It is usually present for life (Hollinger and Liang, 2001).

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2.5.3 Hepatitis C Viral Infection

2.5.3.1 Acute Hepatitis C Infection

Hepatitis C infection causes acute symptoms in 15% of cases (Maheshwari *et al*., 2008). Symptoms are generally mild and vague, including a decreased appetite, fatigue, nausea, muscle or joint pains and weight loss (Wilkins *et al*., 2010). Rarely does acute liver failure result (Bailey, 2010). Most cases of acute infections are not associated with jaundice (Springer, 2011).

2.5.3.2 Chronic Hepatitis C Infection

Chronic hepatitis C infection is defined as the presence of detectable viral replication for at least six months (Springer, 2011). Chronic hepatitis C can be associated with fatigue (Ray *et al*., 2009) and mild cognitive problems (Forton *et al*., 2005). Chronic infection after several years may cause cirrhosis or liver cancer (Rosen, 2011). About 80% of persons exposed to the virus develop a chronic infection (Nelson *et al*., 2011). The liver marker enzymes are normal in about 53% HCV cases (Nicot *et al.,* 2011). Late relapses after apparent cure have been reported, but these can be difficult to distinguish from re infection (Nicot *et al.,* 2011).

Fatty changes to the liver occur in about half of those infected and are usually present before cirrhosis develops (El-Zayadi, 2008; Paradis and Bedossa, 2008). Usually (80% of the time) this change affects less than a third of the liver mass (El-Zayadi, 2008). Worldwide, hepatitis C viral infection is the cause of 27% of cirrhosis cases and 25% of hepatocellular carcinoma (Alter, 2007). About 10–30% of those infected develop cirrhosis over 30 years (Rosen, 2011; Wilkins *et al*., 2010). Cirrhosis is more common

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in persons also infected with hepatitis B, schistosoma, HIV and in alcoholics (Wilkins *et al*., 2010). In people with hepatitis C infection, excess alcohol increases the risk of developing cirrhosis hundred-fold (Mueller *et al*., 2009). Those who develop cirrhosis have a twenty-fold greater risk of hepatocellular carcinoma (Mueller *et al*., 2009). Liver cirrhosis may lead to portal hypertension, ascites (accumulation of fluid in the abdomen), easy bruising or bleeding, varices (enlarged veins, especially in the stomach and esophagus), jaundice, and a syndrome of cognitive impairment known as hepatic encephalopathy (disorder of mental activity, neuromuscular function and consciousness) (Ozaras and Tahan, 2009). Ascites occurs at some stage in more than half of those who have chronic infection (Zaltron *et al*., 2012).

2.6 Liver Damage Markers

Liver damage markers are groups of blood markers that give information about the state of a patient's liver (Lee and Mary, 2009). These markers include prothrombin time, albumin, bilirubin, liver transaminases (aspartate aminotransferase and alanine aminotransferase). The others are alkaline phosphatase (ALP) and Gamma glutamyl transpeptidase (GGT). These are useful markers of liver injury in a patient with some degree of intact liver function (Johnston, 1999; McClatchey and Kenneth, 2002). Most liver diseases cause only mild symptoms initially. Hepatic (liver) involvement in some diseases can be of crucial importance. This testing is performed on a patient's blood sample. Some tests are associated with functionality (e.g., albumin), some with cellular integrity (e.g., transaminase), and some with conditions linked to the biliary tract

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(gamma-glutamyl transferase and alkaline phosphatase). Several biochemical tests are useful in the evaluation and management of patients with hepatic dysfunction. These tests can be used to detect the presence of liver disease, distinguish among different types of liver disorders, gauge the extent of known liver damage, and follow the response to treatment. Some or all of these measurements are also carried out (usually about twice a year for routine cases) on those individuals taking certain medications, such as anticonvulsants, to ensure the medications are not damaging the person's liver.

2.6.1 Albumin

Albumin is a protein made specifically by the liver. It is the main constituent of total protein (the remaining from globulins). Albumin levels are decreased in chronic liver disease, such as cirrhosis. It is also decreased in nephrotic syndrome, where it is lost through the urine. The consequence of low albumin can be edema since the intravascular oncotic pressure becomes lower than the hydrostatic pressure. An alternative to albumin measurement is prealbumin, which is better at detecting acute changes (half-life of albumin and prealbumin is about 2 weeks and 2 days, respectively) in the synthetic function of the liver.

2.6.2 Alanine Aminotransferase

Alanine aminotransferase (ALT) is an enzyme produced by the liver cells in the highest amounts. Low levels of ALT are normally found in the blood. Alanine aminotransferase is located in the hepatocytes. Injury to the liver results in the release of the enzyme into

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the blood which causes ALT levels to increase in the blood. The alanine aminotransferase (ALT) test is used to evaluate liver function.

2.6.3 Aspartate Aminotransferase

Aspartate aminotransferase is similar to alanine aminotransferase (ALT) in that it is another enzyme associated with liver parenchymal cells. It is raised in acute liver damage, but is also present in red blood cells, cardiac and skeletal muscle, so is not specific to the liver. The ratio of AST to ALT is sometimes useful in differentiating between causes of liver damage (Nyblom, 2006). Elevated AST levels are not specific for liver damage, and AST has also been used as a cardiac marker (Nyblom, 2006).

2.6.4 Total Bilirubin

Measurement of total bilirubin includes both unconjugated and conjugated bilirubin. Unconjugated bilirubin is a breakdown product of heme (a part of hemoglobin in red blood cells). Heme can also come from myoglobin, found mostly in muscle, cytochromes, found mostly in mitochondria, catalase, peroxidase, and nitric oxide synthase. Bilirubin is very hydrophobic and mainly transported bound to albumin circulating in the blood. In addition high-concentration hydrophobic drugs (certain antibiotics, diuretics) and high free fatty acids can cause elevated unconjugated bilirubin. The liver is responsible for clearing the blood of unconjugated bilirubin, and about 30% of it is taken up by a normal liver on each pass of the blood through the liver by the following mechanism: Bilirubin is taken up into hepatocytes, 'conjugated' (modified to make it water-soluble) by UDP-glucuronyl-transferase, and secreted into the bile by canalicular multispecific organic anion transporter (CMOAT), which is

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excreted into the intestine. In the intestine, conjugated bilirubin may be metabolized by colonic bacteria, eliminated, or reabsorbed. Metabolism of bilirubin into urobilinogen followed by reabsorption of urobilinogen accounts for the yellow color of urine, as urine contains a downstream product of urobilinogen. Further metabolism of urobilinogen into stercobilin while in the bowels accounts for the brown color of stool. Thus, having white or clay-colored stool is an indicator for a blockage in bilirubin processing and thus potential liver dysfunction or cholestasis.

2.6.5 Gamma-Glutamyl Transferase

Gamma-glutamyl transferase (GGT) is an enzyme that transfers gamma-glutamyl functional groups. It is found in many tissues, the most notable one being the liver, and has significance in medicine as a diagnostic marker. Gamma-glutamyl transferase is present in the cell membranes of many tissues, including the kidneys, bile duct, pancreas, gallbladder, spleen, heart, brain, and seminal vesicles (Goldberg, 1980). It is involved in the transfer of amino acids across the cellular membrane (Meister, 1974) and leukotriene metabolism (Raulf *et al.,* 1985).

Gamma-glutamyl transferase is predominantly used as a diagnostic marker for liver disease in medicine; latent elevations in GGT are typically seen in patients with chronic viral hepatitis infections often taking 12 months or more (Lim *et al.,* 2007). Gamma-glutamyl transferase is elevated by large quantities of alcohol ingestion. However, determination of high levels of total serum GGT activity is not specific to alcohol intoxication (Lamy *et al.,* 1974) and the measurement of selected forms of the enzyme offer more specific information (Franzini *et al,.* 2006). The mechanism for this

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elevation is unclear. Alcohol consumption increases GGT production by inducing hepatic microsomal production, or causes the leakage of GGT from hepatocytes (Barouki *et al.,* 1983). Numerous drugs can raise GGT levels, including barbiturates and phenytoin (Rosalki *et al.,* 1971). Elevated levels of GGT can also be due to congestive heart failure (Ruttmann *et al.,* 2005).

2.7 Aflatoxin and Viral Hepatitis Infection

Hepatitis B virus has double stranded DNA. The virus primarily interferes with the functions of the liver by replicating in the hepatocytes. The virus binds to the host cell and are subsequently internalized by endocytosis (Coffin *et al*., 2011). Liver damage is initiated and mediated by the Cytotoxic T Lymphocyte (CTLs); platelets activated at the site of infection may facilitate the accumulation of CTLs in the liver (Iannacone *et al*., 2005). Hepatitis B viral infection is estimated to be the cause of 30% of cirrhosis and 53% of hepatocellular carcinoma (HCC) worldwide (Perz, 2006).

Hepatitis virus (C) virus replicates mainly in the hepatocytes where it is estimated that each infected cell produces approximately fifty virions per day (Bartenschlager and Lohmann, 2000). Once inside the hepatocyte the virus takes over portions of the intracellular machinery to replicate (Lindenbach and Rice, 2005).

The risk of liver cancer in individuals exposed to chronic viral hepatitis B virus infection and aflatoxin is greater than the risk in individuals exposed to aflatoxin alone (Groopman *et al*., 2008; Liu and Wu, 2010). These two hepatocarcinoma risk factors (aflatoxin and hepatitis B virus) are prevalent in developing countries worldwide (Groopman *et al*., 2008). Aflatoxin also appears to have a synergistic effect on hepatitis

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C virus-induced liver cancer, although the quantitative relationship is not as well established as that for aflatoxin and hepatitis B virus in inducing hepatocarcinoma (Liu and Wu, 2010; Kirk *et al*., 2006; Wild and Montesano, 2009). Aflatoxins have been found to cause an increase in liver damage markers, serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST) in a study involving mice induced with aflatoxin B1 (Anamika *et al*., 2013). In another study involving children in Gambia, blood samples were analyzed for aflatoxin-albumin (AF-alb) with serum alanine aminotransferase (ALT) as markers of liver damage. The study found a significant positive correlation between blood aflatoxin-albumin and serum alanine aminotrasferase (Wild *et al*., 1993). In China, 10% of all adult deaths were due to liver cancer (Wang *et al*., 1996). To minimize the risk of liver cancer, it is critically important that exposure of HBV- and HCV-infected persons to aflatoxin is minimized.

2.7.1 Epidemiology of Aflatoxins and Hepatocarcinoma

Epidemiological studies in the 1970s and 1980s in sub-Saharan Africa and south-east Asia reported correlations between aflatoxin levels in food crops and hepatocarcinoma rates (IARC, 1993). However, majority of this early work did not take account of HBV infection and aflatoxin exposure (IARC, 1993). In the1990s significant advances were made in understanding the role of aflatoxins as a risk factor for hepatocarcinoma, particularly in relation to joint effects with chronic HBV infection. Progress came from two principal sources: Prospective cohort studies and analyses of the molecular pathology of hepatocarcinoma (HCC) specimens, both of which involved aflatoxin biomarkers. Two key cohort studies in Asia and a large case-control study in Africa

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employed biomarkers (urinary aflatoxin) metabolites and blood aflatoxin–albumin adducts to improve individual aflatoxin assessment. The study showed significant interactions with chronic hepatitis B viral (HBV) infection in relation to HCC risk (Qian *et al*., 1994). Both studies in Asia, Shanghai (Qian *et al*., 1994; Wang *et al*., 1996; Ross *et al*., 1992) reported a more than multiplicative interaction between the two risk factors (aflatoxin and hepatitis B viral infection). In a follow-up of the Taiwan cohort, Sun and Co-workers (2001) showed that in hepatitis B surface antigen (HBsAg) carriers, those with detectable aflatoxin–albumin adduct were more likely to develop hepatocarcinoma. In similar studies restricted to individuals chronically infected with hepatitis B virus revealed increased hepatocarcinoma risk in persons also positive for aflatoxin bio-markers (Yu *et al*., 1997; Chen *et al*., 1997).

In a case-control studies by Omer *et al*., (2004) conducted in Sudan, reported peanut consumption (as a surrogate for aflatoxin exposure) was associated with HCC and showed a more than additive interaction with hepatitis B virus.

In comparison to the study of aflatoxins and interaction with HBV there has been little focus on the potential for interaction with hepatitis C viral (HCV) infection (Sun *et al*., 2002; Chen *et al*., 2007). A study in Taiwan suggested that aflatoxin–albumin adducts were associated with advanced liver disease in individuals infected with HCV (Chen *et al*., 2007).

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2.7.2 Potential Mechanisms of Interaction of Aflatoxin and Viral Hepatitis

Sero-epidemiological studies have clearly shown that in the natural history of hepatitis B viral infection, two factors are of importance in determining the risk of hepatocarcinoma. These are the age at primary infection and the presence of hepatitis B envelope antigen (HBeAg) or hepatitis B viral DNA, biomarkers of active viral replication, in patients with chronic active hepatitis (Yang *et al.,* 2002). These observations are important when exploring potential mechanisms of interaction with aflatoxins. The development of hepatocarcinoma results from the accumulation of various genetic changes at different stages of liver carcinogenesis (Lee and Thorgeirsson, 2006). Chronic liver injury and regenerative hyperplasia, resulting from hepatitis B viral infection, are critical to the development of liver cancer (Hussain *et al*., 2007; Dunsford *et al*., 1990). It is possible that aflatoxin-induced DNA adducts are fixed as mutations due to the HBV related increase in cell proliferation and hyperplasia, thus promoting the clonal expansion of mutant cells (Wu *et al*., 2007). Hepatitis B virus may also alter the hepatic expression of aflatoxin metabolising enzymes and consequently the extent to which aflatoxins bind to DNA. Studies in HBV transgenic mice revealed an induction of specific cytochrome P450s (CYP) in association with liver injury induced by overexpression of hepatitis B surface antigen (Chemin *et al*., 1996; Kirby *et al*., 1994). The effects of liver injury are not limited to cytochrome P450 enzymes. An increase in glutathione S-transferase Pi (GST-pi has significance in the diagnosis of cancers as it is expressed abundantly in tumor cells) was also observed in the HBV transgenic mice (Chemin *et al.,* 1999). In human liver, GST activity was lower in the presence of HBV DNA (Zhou *et al*., 1997) suggesting viral infection may

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compromise the ability of hepatocytes to detoxify chemical carcinogens. Overall, the effects of HBV infection on aflatoxin metabolism are likely to be complex.

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CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

Ejura-Sekyedumase district in the Ashanti Region is located in the middle belt of Ghana. The climatic conditions are those of savanna transition agro-ecological zone. The mean annual rainfall is 1400 mm which is received in two peak season but sometimes in a unimodal pattern (Dedzo, 1998). Temperatures are uniformly high with the mean annual maximum value of about 32 ^{o}C and the minimum approximately 20 ^{o}C. The relative humidity values are high during the rainy season with the mean monthly values ranging between 80-88% in the morning and decreasing to 70-75% by mid day. During the dry season the humidity value drops to approximately 75% in the morning and 45% by mid day (Dedzo, 1998).

3.2 Minimum Sample Size Calculation

$$N = \frac{\left[u\sqrt{\pi_1(1-\pi_1)+\pi_2(1-\pi_2)} + v\sqrt{(\pi_1+\pi_2)\left(1-\frac{(\pi_1+\pi_2)}{2}\right)} \right]^2}{(\pi_1-\pi_2)^2}$$

Where:

π_1 = Proportions of cases exposed

π_2 = Proportion of controls exposed

μ = Sample proportions of cases

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v = Sample proportion for controls

N = Sample size per group

The sample size was determined at the 95% confidence level and power of 90%, with case control ratio of 1:1 and exposure of risk viral hepatitis among cases to be 18%. A minimum sample size per group = 55. For this study, a total of 55 cases and 55 controls were recruited.

3.3 Inclusion and Exclusion Criteria

Inclusion Criteria

- Adults of 18yrs and above.
- Informed consent signed.

Exclusion Criteria

- Children below 18yrs
- Pregnant Women
- Lactating Mothers

3.4 Ethical Consideration

Ethical approval was given by the Ethical and Protocol Review Committee of the College of Health Sciences, University of Ghana, Legon with protocol identification number: MS-Et/M.7-P 3.6/2014-2015. All data were handled confidentially.

3.5 Consent Form

Informed written consent was obtained from all participants in accordance with the human experimental guidelines of the University of Ghana. Confidentiality was

observed throughout the research process and the data made accessible only to the research team.

3.6 Sampling Methods

The study employed the modified World Health Organization (WHO) cluster sampling method to select eligible subjects. The Ejura-Sekyedumase district community was segmented into four clusters (communities) by natural/geographical boundaries. A cluster was chosen by a simple random sampling technique and all eligible subjects within households who consented were included. Homes in the community have standard house numbering systems, and a household within a home was numbered serially. If more than one household exist within a home, then the first household interviewed was designated as house number/001, the second household interviewed was designated as house number/002 and serially if there are more eligible households within the same house. All households within a selected cluster were eligible for inclusion. One adult member of each household within a home was interviewed, and this continued until the number of subjects to be screened i.e. 500 was obtained. A hundred and twenty five subjects were selected from each community.

3.6.1 Sample Selection

A total of five hundred (500) subjects including three hundred and twenty males (320) and one hundred and eighty females (180) who consented were screened for viral hepatitis B surface antigen and hepatitis C viral antibodies based on the prevalence rate of more than 10% of hepatitis B infection and 25% prevalence rate of hepatitis C

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infection in the Ejura-Sekyedumase district (Ejura-Sekyedumase District Hospital Annual Report, 2014). Forty three (43) subjects (33 males and 10 females) who tested positive for viral hepatitis B surface antigen were selected as group A with age range from 18-47years. Based on the initial findings of 43 subjects who tested positive for hepatitis B surface antigen, forty three (43) subjects (30 males and 13 females) who showed negative to the test were selected as control group (group B) with age range 18-47 years to match. Twelve subjects (7 males and 5 females) who tested positive for hepatitis C viral antibodies were also selected as group C with age range 18-47 years. Based on the initial findings, twelve subjects (7 males and 5 females) who tested negative for hepatitis C antibodies were selected as control group (group D) with age range 18-47 years to match.

3.7 Study Design

The study was a community based case-control study involving 55 cases (HBV and HCV) and 55 controls from the Ejura-Sekyeduamse district in the Ashanti Region of Ghana.

3.8 Materials

Vicam AflaTest kits were used according to the Association of Official Analytical Chemists' method (AOAC) for aflatoxin in AOAC official method 993.31, V1 series 4. Sodium chloride (NaCl) and methanol (HPLC) grade were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). AflaTest columns were purchased from VICAM

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(Watertown, MA, USA). All other chemicals and reagents used were of highest purity available and commercially purchased.

3.8.1 Specimen Collection and Processing

3.8.1.1 Urine Collection

Spot urine samples were successfully obtained from 110 adults with a sterile specimen cup at the Ejura-Sekyedumase district hospital laboratory. The urine samples were frozen and transported to Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana, Legon, in a leak free cool box. They urine were kept frozen at -20ºC until analysis of afatoxin M_1 (AFM_1) levels at NMIMR, University of Ghana, Legon.

3.8.1.2 Blood Collection

Whole blood (5 mL) was collected from each study participant by venipuncture into a labeled blood clotter tube at the Ejura-Sekyedumase district hospital laboratory. The blood was centrifuged at 2500 rpm for 10 minutes to collect the serum which was stored in aliquots in freezing vials, labeled with participant's name and identification number. The serum samples were frozen and transported to NMIMR as described in section 3.8.1.1 above.

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3.9 Sample Analysis

3.9.1 Urinary Aflatoxin M1 (AFM_1)

Aflatoxin M_1 levels in 110 urine samples were analyzed with immunoaffinity column purification (AflaTest) followed by HPLC-flourescence detection at 365 nm excitation and 425 nm emission wavelengths. Each urine sample (5 mL) was acidified with 0.5 ml of 1.0.M ammonium formate (pH 4.5) and diluted to a volume of 10 mL with deionised water. Samples were then allowed to flow through the immunoaffinity columns by gravity at a flow rate of 1-2 drops per second. Columns were washed with phosphate buffer saline (PBS) and deionised water before elution of AFM_1 with 80% methanol. The eluants were then dried under nitrogen gas and resuspended in methanol: ammoniumformate (ratio 1:1) solution for analysis using a Shimadzu HPLC system with fluorescence detection (Shimadzu Corporation, Japan). A 250 x 4.6 mm LiCrospher RP-18EC end capped column with a pore size of 100 A and a particle size of 5 µm (Alltech) was used to resolve aflatoxin metabolites. The mobile phase was 22% ethanol in deionised water buffered with 20 mM ammonium formate (pH 3.0). Samples (100 µL) were injected at an elution rate of 1mL/min. The limit of detection was 0.5 pg AFM_1/mL urine. Analysis of AFM_1 was done at the Clinical laboratory department of the Noguchi Memorial Institute for Medical Research (NMIMR)

3.9.2 Liver Damage Markers

The levels of liver damage markers in the serum; alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (ALB) and gamma-glutamyl transferase (GGT) were measured using an automated colorimetric method by Flexor E, endpoint

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automation, Vital Scientific, Netherlands. Analysis of liver damage markers was done at the Clinical Pathology Department, Noguchi Memorial Institute for Medical Research.

3.9.3 Viral Hepatitis B and C Screening

Screening of viral hepatitis B surface antigen and hepatitis C antibodies were done using Clinogen rapid kit from Warehouse/Robin Willis Way, Windsor SL4 2PX, United Kingdom, with specificity of 98.5%, sensitivity 99.0% and accuracy of 98.5%. A volume of 50 μL aliquot of serum each was used for hepatitis B surface antigen and hepatitis C antibody screening. Screening of hepatitis B viral surface antigen and hepatitis C viral antibodies was done at the Clinical Pathology Department of the Noguchi Memorial Institute for Medical Research.

3.9.4 Hepatitis B Viral Profile

Hepatitis B core antibody (HBcAb), Hepatitis B envelope Antigen (HBeAg), Hepatitis B envelope Antibody (HBeAb) and Hepatitis B surface Antibody (HBsAb) were determined on samples which tested positive for hepatitis B surface antigen using a rapid assay from Clinogen Laboratories. The test results were read within 30 minutes. The test was done at the Clinical Pathology Department of the Noguchi Memorial Institute for Medical Research.

3.9.5 Statistical Analysis

The data from aflatoxin M1, liver damage markers and results in both hepatitis (B and C) positive and negative blood samples were analysed using simple descriptive statistics

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by the International Business Machine (IBM) Statistical Package for the Social Sciences, version 20, which looked at the range, the mean (n) and the standard deviation (SD). Statistical significance was compared using the t-test analysis. $P < 0.05$ and Pearson Product Moment correlation coefficient (r), $r = 0.900$ were considered to be significant and of association respectively.

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CHAPTER FOUR

4.0 RESULTS

The study recruited five hundred participants (320 males and 180 females) who were screened for both hepatitis B viral surface antigen and hepatitis C viral antibodies. Out of the 500 subjects screened 43 (8.6%) which includes 33 (6.6%) males and 10 (2%) females tested positive for hepatitis B surface antigen only. Four hundred and fifty seven (457) subjects which represent 91.4% of the total subjects screened tested negative for hepatitis B surface antigen. Twelve (2.4%) out of the 500 subjects screened were positive for hepatitis C viral antibodies only, which consisted of 7 (1.4%) males and 5 (1%) females. Four hundred and eighty eight (488) subjects tested negative for viral hepatitis C antibodies (table 4.1).

Based on the initial findings of 43 subjects (group A) who tested positive for hepatitis B surface antigen, forty three (43) subjects (30 males and 13 females) who showed negative to the test were selected as control (group B) to match group A (table 4.3). Four subjects (0.8%) tested positive for hepatitis B envelope antigen (table 4.2). Twelve subjects (7 males and 5 females) who tested positive for hepatitis C antibodies were also selected as group C. Based on the initial findings of 12 subjects who tested positive for hepatitis C antibodies, twelve (12) subjects (7 males and 5 females) who showed negative to the test were selected as control group (group D) to match group C (table 4.4).

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4.1 Gender of study population screened for hepatitis B and C.

Table 4.1 shows the data of the study population screened for viral hepatitis B and C. A total number of 500 subjects were screened. Forty three (43) subjects tested positive for viral hepatitis B whiles twelve subjects tested positive for viral hepatitis C.

Table 4.1 Gender distribution of study population screened for viral hepatitis

	Hepatitis B		Hepatitis C	
Gender	**Negative**	**Positive**	**Negative**	**Positive**
Male	287 (57.4)	33 (6.6)	313 (62.6)	7 (1.4)
Female	170 (34)	10 (2)	175 (35)	5 (1)
Total	**457 (91.4)**	**43 (8.6)**	**488 (97.6)**	**12 (2.4)**

n(percentage) **N=500**

4.2 Gender and age data of hepatitis B surface antigen positive subjects

Table 4.2 shows gender and age data of hepatitis B surface antigen positive. Forty three subjects (33 males and 10 females) based on age were selected as group A (hepatitis B surface antigen positive) for the study. The 43 selected were also positive for hepatitis B core antibody (HBcAb). Four (3males and a female) out of the 43 were also positive for hepatitis B envelope antigen (HBeAg) whiles none of the subjects showed positive for hepatitis B surface antibodies (HBsAb). Out of the 43 who tested positive for hepatitis B surface antibodies, thirty three (26 males and 7 females) tested positive for hepatitis B envelope antibodies (HBeAb).

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Table 4.2 Gender and age distribution data of hepatitis B surface antigen positives (group A)

Age group (yrs)	Number of HBsAg Positives		Number of HBsAb Positives		Number of HBcAb Positives		Number of HBeAg Positives		Number of HBeAb Positives	
	M	F	M	F	M	F	M	F	M	F
18-23	3	2	0	0	3	2	1	0	3	2
24-29	11	5	0	0	11	5	1	1	7	2
30-35	13	2	0	0	13	2	1	0	11	2
36-41	4	0	0	0	4	0	0	0	4	0
42-47	2	1	0	0	2	1	0	0	1	1
Total	**33**	**10**	**---**	**----**	**33**	**10**	**3**	**1**	**26**	**7**

M= Males, F= Females

The average age was 30 years with highest frequencies occurring between subjects aged 30-35 years for males and 24-29 years for females.

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4.3 Data of hepatitis B surface antigen negative subjects

Table 4.3 shows data of hepatitis B surface antigen negative (group B). Forty three subjects (30 males and 13 females) based on age were selected as control group for the study in order to match the hepatitis B surface positive group.

Table 4.3 Gender and age distribution data of hepatitis B surface antigen negative subjects (Group B)

Age group (yrs)	Number of HBsAg Negative	
	Male	Female
18-23	4	2
24-29	8	7
30-35	4	2
36-41	8	1
42-47	6	1
Total	**30**	**13**

The average age was 29 years with highest frequencies occurring between ages 24-29 years for males and 36-41 years for females.

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4.4 Data of hepatitis C antibody positive subjects

Table 4.4 shows data of hepatitis C antibody positive subjects (group C) and control (group D). Twelve adults (7 males and 5 females) who tested positive for hepatitis C antibodies where selected as group C, whiles twelve adults (7 males + 5 females) who tested negative for hepatitis C viral antibodies were selected as control (group D) to match the hepatitis C viral antibodies positive group (C) for the study.

Table 4.4 Gender and age distribution data of hepatitis C viral antibody positive (group C) and control (group D)

Age group (yrs)	Hepatitis C positive		Control	
	Number of viral hepatitis C Positive		Number of viral hepatitis C Negative	
	Male	Female	Male	Female
18-23	1	1	2	1
24-29	0	2	1	2
30-35	3	1	2	1
36-41	2	1	0	0
42-47	1	0	2	1
Total	**7**	**5**	**7**	**5**

The average age was 32 years with highest frequencies occurring between ages 30-35 years for males and 24-29 years for females(group C). The highest frequencies occurred between the ages of 18-23; 30-35 and 42-47 years (males) in group D, whiles the females (group D) had the highest frequencies of 24-29years.

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4.5 Results of urinary AFM_1 and liver damage markers of hepatitis B surface antigen positive subjects

Table 4.5 shows the results of urinary AFM_1 values and liver damage markers of group A (hepatitis B surface antigen positive) and group B (hepatitis B surface antigen negative) subjects. The average urinary $AFM_{1,}$ serum AST, ALT, albumin and GGT for HBsAg positive subjects were 5255.0 pg/dL, 41.7 U/L, 25.6 U/L, 40.3 g/L and 46.2 U/L respectively. The average values of urinary $AFM_{1,}$ serum AST, ALT, albumin and GGT for HBsAg control group were 374.8 pg/dL, 38.4 U/L, 19.2 U/L, 40.4 g/l and 22.4 U/L respectively (table 4.5). There was significant difference in the urinary AFM_1 concentration (5255.0 pg/dL) between the HBsAg positive subjects and the control subjects (374.0 pg/dL) (P= 0.001). There was no significant difference in the levels of serum ALT, AST, and albumin concentrations between the HBsAg positive and the control group. There was significant difference (p=0.001) in serum GGT concentrations between the HBsAg positive (mean GGT= 46.1 U/L) and the control group (mean GGT= 12.1 U/L).There was a 14 fold increase of urinary AFM_1 in hepatitis B surface antigen positive subjects (group A) compared to the control (group B) (table 4.5).

Table 4.5 Results of urinary AFM_1 and liver damage markers in hepatitis B surface antigen (HBsAg) positive subjects and controls

Markers	HBsAg Positive (group A)		HBsAg Negative (group B)		t-Test
	Mean±SD	Range	Mean±SD	Range	P-value
AFM_1 (pg/dL)	5255.0±2757.9	768-12427	374.8±227.7	61.3-717.6	0.001*
ALT (U/L)	25.6±16.1	5.4-70	19.0±11.4	2-49	0.066
AST (U/L)	41.8±27.6	13.5-153	37.8±16.4	6.9-67	0.367
ALB (g/L	40.3± 7.7	19.1-54.3	40.4±6.3	26-53.7	0.660
GGT (U/L)	46.1±26.4	5.9-215	22.4±12.1	5.2-49.3	0.021*

Data is presented as mean±SD. There was a significant difference in the mean AFM_1 and GGT of group A and group B ($p^* < 0.05$). Normal ranges: AFM_1=0.5 ug/kg (FAO, 2003). AST=13-60 U/L, ALT=6-54 U/L, GGT=6-71 U/L and ALB=32.7-49.8 g/L. (Dosoo *et al.*, 2012).

4.6 Results of urinary AFM_1 values and liver damage markers in hepatitis B envelope antigen positive

Table 4.6 shows results of urinary AFM_1 values and liver damage markers of hepatitis B envelope antigen (HBeAg) positive subjects. Four adults in the group A, tested positive for HBeAg.

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Table 4.6 Results of urinary AFM_1 and liver damage markers of hepatitis B envelope antigen (HBeAg) positive subjects

Liver Damage Markers	Mean	SD
AFM_1(pg/dL)	6536.7	± 5558.5
AST(U/L)	78.8	± 55.8
ALT(U/L)	58.0	± 12.4

Normal ranges: AFM_1=0.5 ug/kg (FAO, 2003). AST=13-60 U/L, ALT=6-54 U/L, GGT=6-71 U/L and ALB=32.7-49.8 g/L. (Dosoo *et al.*, 2012).

4.7 Results of urinary AFM_1 and liver damage markers in hepatitis C antibody positive (Group C)

Table 4.7 shows the results of urinary AFM_1 values and liver damage markers in subjects positive for hepatitis C virus (HCV) antibodies (group C) and the controls (group D). The average values of urinary $AFM_{1,}$ AST, ALT, albumin and GGT for hepatitis C positive adults were 426.7 pg/dL, 48.8 U/L, 19.7 U/L, 38.2 g/L and 27.8 U/L respectively. The average values of urinary $AFM_{1,}$ Serum AST, ALT, albumin and GGT for hepatitis C negative subjects were 359.7 pg/dL, 36.3 U/L, 19.3 U/L, 39.3 g/L and 24.5 U/L (table 4.7). There was no significant difference in the urinary AFM_1 mean concentration (426.7 g/dL) between HCV antibody positive subjects and the urinary AFM_1 mean concentration (359.7 g/dL) of their controls. There was no

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significant difference in the serum ALT, AST, albumin and GGT concentrations between the Hepatitis C viral antibody positive and the control group.

Table 4.7 Results of urinary AFM_1 and liver damage markers between hepatitis C viral antibodies positive subjects (group C) and controls (group D)

Markers	HCV Positive (Group C)		HCV Negative (Group D)		t-Test
	Mean	Range	Mean	Range	P-value
AFM_1 (pg/dL)	426.7±237.0	21.3-720.9	359.7±259.2	61.3-717.6	0.515
ALT (U/L)	19.7±16.1	7.6-31.6	36.3±10.1	2-36.1	0.931
AST (U/L)	48.8±28.5	7.2-72.7	19.3±11.3	27-51.9	0.214
ALB (g/L)	38.2±5.1	31.8-44.6.	39.3±6.0	26-49.9	0.610
GGT (U/L)	27.8±12.6	4.0-45	24.5±14.0	5.2-49.0	0.544

There was no significant difference between AFM_1 and liver damage markers of group C and group D. $P < 0.05$ was considered significant. N=12.
Normal ranges: AFM_1=0.5 ug/kg (FAO, 2003). AST=13-60 U/L, ALT=6-54 U/L, GGT=6-71 U/L and ALB=32.7-49.8 g/L. (Dosoo *et al.*, 2012).

4.8 Correlation between AFM_1 and Liver Damage Markers

Figure 4.1(a, b, c and d) shows a correlation graph of urinary AFM_1 vrs liver damage markers in hepatitis B surface antigen (HBsAg) positive subjects.

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Figure 4.1a shows a correlation graph of urinary AFM_1 vrs serum AST of hepatitis B surface antigen (HBsAg) positive subjects. There was no association between AFM_1 and AST of hepatitis B surface antigen positive subjects (r = - 1.0).

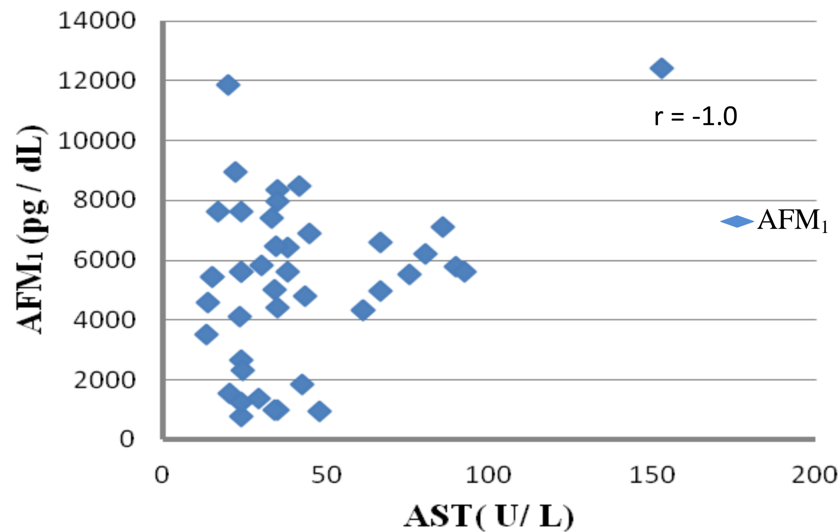

Figure 4.1a. Association between urinary AFM_1 values and serum AST values in HBsAg positive subjects (N=43). There was no association between urinary AFM_1 and serum AST of HBsAg positive subjects (r = -1.0).
r = 0.9: strong positive association, r = 0.2: weak association and r = - 0.9: strong negative association.

Figure 4.1b shows a correlation graph of urinary AFM_1 vrs serum ALT of hepatitis B surface antigen (HBsAg) positive subjects (group A). There was no association between urinary AFM_1 and serum AST of hepatitis B surface antigen (HBsAg) positive (group A) subjects (r = - 1.8).

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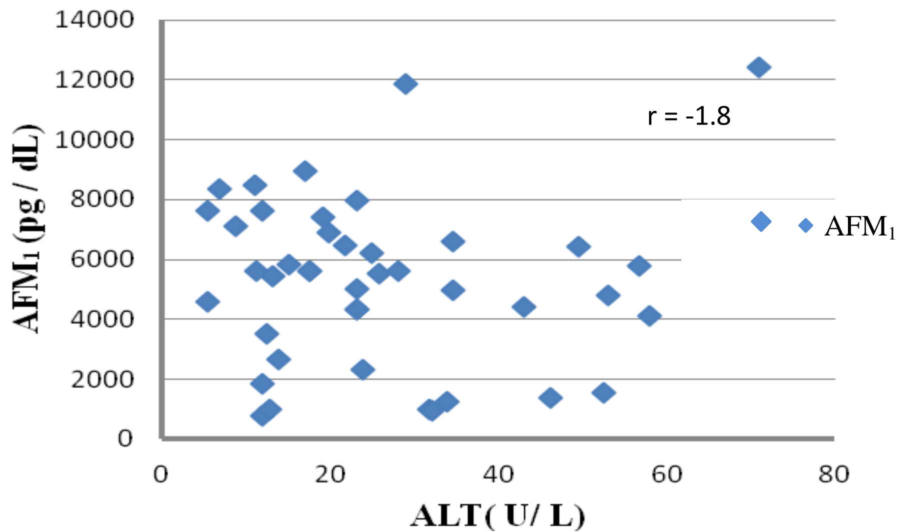

Figure 4.1b. Association between urinary AFM_1 values and serum ALT values in HBsAg positive subjects (N=43). There was no association between urinary AFM_1 and serum ALT of HBsAg positive subjects (r = - 1.8). *r = 0.9: strong positive association, r = 0.2: weak association and r = - 0.9: strong negative association.*

Figure 4.1 c shows a correlation graph of urinary AFM_1 vrs serum albumin (ALB) of hepatitis B surface antigen (HbsAg) positive subjects. There was no association between urinary AFM_1 and serum albumin of hepatitis B surface antigen (HBsAg) positive subjects (r = -2.5).

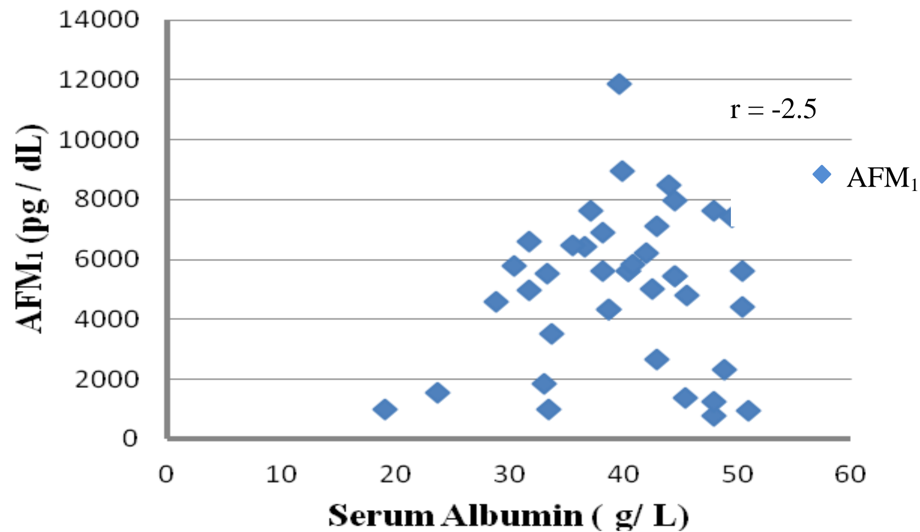

Figure 4.1c. Association between urinary AFM_1 values and serum ALB values in HBsAg positive subjects (N=43). There was no association between urinary AFM_1 and serum ALB of HBsAg positive subjects (r = -2.5).
r = 0.9: strong positive association, r = 0.2: weak association and r = - 0.9: strong negative association.

Figure 4.1d shows a correlation graph of urinary AFM_1 vrs serum gamma-glutamyl transferase (GGT) of hepatitis B surface antigen (HBsAg) positive subjects. There was no association between urinary AFM_1 and serum GGT of hepatitis B surface antigen (HBsAg) positive subjects (r = - 0.8).

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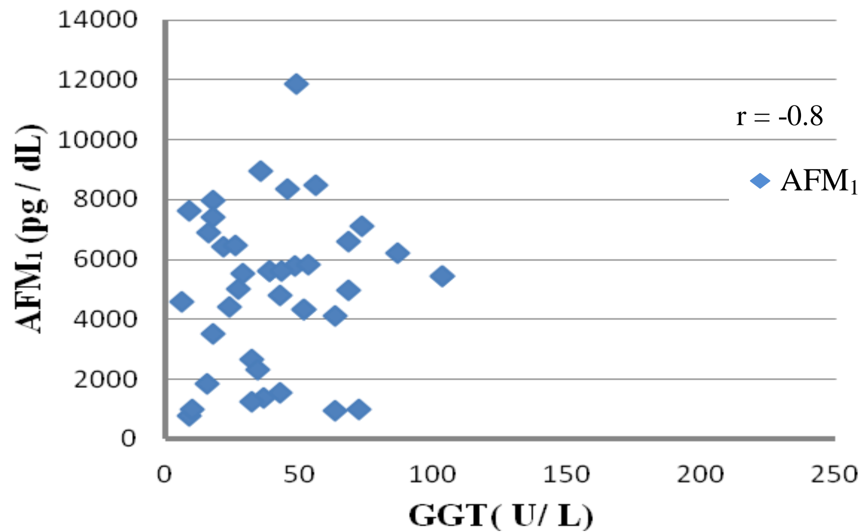

Figure 4.1d. Association between urinary AFM_1 values and serum GGT values in HBsAg positive subjects (N=43). There was no association between urinary AFM_1 and serum GGT of HBsAg positive subjects (r = - 0.8).

r = 0.9: strong positive association, r = 0.2: weak association and r = - 0.9: strong negative association.

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CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATION

A significant association between aflatoxin exposure and human liver diseases has been reported in endemic areas in Sub-Sahara Africa, East and Southeast Asia which are highly exposed to aflatoxin and liver diseases (Colombo, 1992; Qian *et al*., 1994). Rates of hepatitis B viral (HBV) positivity in The Gambia, China and Guinea are 15% (Allen *et al*., 1992), 14-20% (Wang *et al*., 2001), and 10% (Diallo *et al*., 1995), respectively. The effect of hepatitis B viral infection and the efficiency with which aflatoxin is detoxified is affected by biological exposure to aflatoxin (Kelly *et al*., 1997).

Several factors are known to affect the progression of hepatitis C viral related diseases (Fattovich *et al*., 2004). However, it remains unclear whether aflatoxin exposure is an associated risk factor for advanced liver disease including liver cirrhosis in patients with chronic hepatitis C (Boutrif, 1998). Two cohort studies in South-East Asia, have demonstrated an interaction between hepatitis B virus and aflatoxins in determining hepatocarcinoma risk. Both studies used biomarkers of aflatoxin M1 to classify individuals for exposure status and showed significant association with chronic infection in relation to hepartocarcinoma (Qian *et al*., 1994; Wang, 1996). This study measured the level of exposure of aflatoxin and liver damage markers in adults with viral hepatitis infection.

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The levels of urinary AFM_1 concentrations found in subjects in the present study (both control and hepatitis B surface antigen positive) ranged from 61.3 to 12,427 pg/dL (table 4.5). Alessandra de Cássia Romero *et al.,* (2009) detected AFM_1 in urine samples of 45 subjects in the range of 180 to 3,990 pg/ dL in a study involving Brazilian population. The results showed a lower incidence range of urinary AFM_1 concentration as compared to the current study.

Allen and his colleagues (1992) in a study of aflatoxin exposure, malaria and hepatitis B infection in rural Gambia reported that aflatoxin exposure was much lower in the raining season compared to the dry season. The difference in urinary AFM_1 concentration between the findings in the present study and the report by Alessandra de Cássia Romero and his group may have occurred due to seasonal variation of aflatoxin exposure and dietary data which were not investigated in the present study.

The progression of aflatoxin in hepatitis B surface antigen positive (HBsAg+) individuals is substantially higher than in hepatitis B surface antigen negative (HBsAg-) individuals (Henry *et al*., 2002). Thus, reduction of the intake of aflatoxins in populations with a high prevalence of HBsAg positive individuals will have greater impact on reducing liver diseases (Henry *et al*., 2002). A study conducted by Mizrak *et al*., (2004) documented that in viral hepatitis B positive subjects, aflatoxin concentration is significantly higher than hepatitis B surface antigen negative subjects in Turkey.

There was significant difference in aflatoxin M1 concentrations between subjects who were positive for hepatitis B surface antigen (group A) compared to those who tested negative for hepatitis B surface antigen (table 4.5).

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Aflatoxin concentration in hepatitis B surface antigen positive subjects in the current study was higher than in the hepatitis B surface antigen negative subjects (control group). These findings are comparable to the study by Mizrak and his group (2004). A factor in this greater potency of aflatoxin in HBV-positive people is that HBV positivity reduces the person's ability to detoxify aflatoxin (Allen *et al.,* 1992)

Prospective studies in Shanghai, China by Qian *et al*., (1994) showed that urinary excretion of aflatoxin metabolites is a useful indicator for the increased risk of hepatocarcinoma. Exposure of aflatoxin in hepatitis B viral positive subjects increased hepatocarcinoma risk up 7-fold (Qian *et al*., 1994). The current study demonstrated a 14 fold increase of urinary AFM_1 in the hepatitis B surface positive subjects (group A) compared to the hepatitis B surface antigen negative subjects (group B). The two times fold increase in the concentrations of AFM_1 in hepatitis B surface antigen positive subjects in the present study compared to the study by Qian and his group could be due to the concentrations of aflatoxin B1 ingested and the efficiency with which aflatoxin is metabolized and possibly the number of subjects tested (Kelly *et al*., 1997). Although aflatoxin B1 (AFB_1) might contribute to hepatocarcinogenesis by other mechanisms, its role in pathogenesis of hepatocarcinoma is primarily mediated by its effects on chronic hepatitis B viral infection. Various mechanisms of interaction between aflatoxin and HBV in hepatocarcinogenesis have been proposed. It may reflect changes in metabolism of aflatoxin B1 (AFB_1) with coexistence of HBsAg. The most widely accepted is that, hepatitis B viral infection sensitizes hepatocytes to the carcinogenic effects of aflatoxin B_1 (Kew, 2003). This can be done by inducing cytochrome P450

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enzyme that metabolizes AFB_1 to the toxic metabolite AFB_1-8, 9-epoxide (Chemin *et al*., 1999). In other studies, a positive interaction between HBV and AFB_1 seemed to depend on the absence of detoxification enzymes like glutathione-S-transferase, which converts the carcinogenic AFB_1-8,9-epoxide to non-reactive metabolites (Chen *et al*., 1996; Yu *et al*., 1997; Sun *et al*., 2001). Primary prevention, such as vaccination for hepatitis B virus and control of aflatoxin contamination of food, offers strategies for lowering hepartocarcinoma rates.

There was no significant difference in the present study in urinary AFM_1 concentrations between hepatitis C viral antibody positive (group C) the control (group D) (table 4.7). There was no significant difference between liver damage markers of both control group and hepatitis C viral antibody positive group (table 4.7). It remains unclear whether aflatoxin exposure is an associated risk factor for advanced liver disease in patients with chronic hepatitis C. Community-based studies in Taiwan revealed that hepatitis C positive status does not significantly affect temporal variability in aflatoxin B1 (AFB_1)-albumin adducts (Chen *et al*., 1996; Sun *et al*., 2002). In another study conducted in Egypt, a hepatitis C viral (HCV) endemic country, found that the incidence of liver diseases in HCV infected patients is strongly associated with the food and the seeds contaminated with aflatoxins (Hifnawy *et al*., 2004). The study showed that aflatoxin exposure levels were associated with HCV infection and was an independent risk factor for advanced liver disease in patients with HCV infection. Furthermore, the level of aflatoxin exposure was significantly related to ultrasonographic parenchyma scores. These scores reflect the severity of liver fibrosis.

Thus, aflatoxin exposure might be associated with advanced liver fibrosis in patients with chronic hepatitis C (Hifnawy *et al*., 2004). The study participants employed in the current study as hepatitis C positive (group C) have not developed advance stage liver disease therefore the effect of aflatoxin exposure was not as in the advance liver disease state with other related diseases. The biological mechanism underlying the interaction between chronic HCV infection and increased activation of aflatoxin in humans merits further study.

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are commonly measured clinically as a marker for the liver health (Hou *et al*., 2013). Alterations in serum levels of ALT and AST are liver specific and have been considered as a tool for studying varying cell viability and changes in cell membrane permeability (El-Zayadi, 2008). In addition, serum Gamma-glutamyl transferase (GGT) is a prime marker of bile duct epithelial proliferation that is typical of aflatoxicosis (Kramer, 1989). Adedara *et al*., (2010) found that an increase in hepatic alkaline phosphate activity was well supported by the parallel increase in GGT activity following exposure to aflatoxin B1 (AFB_1). Albumin is the main protein made by the liver, circulates in the bloodstream and is affected by liver disorders.

In a study involving children in The Gambia, blood samples were analyzed for aflatoxin-albumin (AF-alb) with serum alanine aminotransferase (ALT) as a marker of liver damage. The subjects were followed up for one year. The study found no association between aflatoxin albumin adduct and ALT (Wild *et al*., 1993). In table 4.5

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in the present study there was no significant difference in ALT activity and urinary AFM_1 HBsAg positive (group A) and urinary AFM_1 HBsAg negative (group B) subjects. However in another study conducted by Mohgah *et al.* (2014) in Egypt, flour mill workers exposed to aflatoxin between 7-38 years had significant difference of GGT between the exposed workers and their controls. However, in this study significant difference in GGT activity was found between control subjects (table 4.5) (hepatitis B surface antigen negative) and hepatitis B surface positive group. This study demonstrated no association between serum ALT, AST, albumin and GGT vrs urinary AFM_1 (Fig 4.1a- 1.4d) in HBsAg positive subjects.

This study agrees with the studies done by Wild and his group (1993) who found no association between aflatoxin and ALT. Also the study done by Morgah and collegues (2014) confirms the significance difference of GGT in the present study. Though longer period of aflatoxin exposure may be required as done by this group, the present study could not confirm the exposure period of aflatoxin in the study subjects.

Gamma-glutamyl transferase is used to evaluate bile duct obstructions. It is also used to screen for chronic alcohol abuse in people who are receiving treatment for alcoholic hepatitis. In contrast to the present study, chronic alcohol abuse and bile duct obstructions were not evaluated. Therefore the significant difference in GGT between the hepatitis B surface antigen positive group and the control group may not be due to aflatoxin only, but other contributing factors as mentioned above.

Increase in liver damage markers in chronic hepatitis B infection may also depend on the chronic phases of hepatitis B viral infection (McMahon, 2005). The immune active phase is characterized by elevated levels of aminotransferases and elevated hepatitis B

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viral DNA level above 2000 IU/mL with the presence of hepatitis B envelope antigen (HBeAg) (McMahon, 2005). Inactive chronic hepatitis B phase is characterized by the absence of HBeAg and normal aminotransferase levels and hepatitis B viral (HBV) DNA<2000 IU/mL (De Franchis *et al*., 1993; Zacharakis *et al*., 2005).

This study recorded majority (39) subjects (out of 43) test group in the inactive chronic hepatitis B phase which could also account for the insignificant level of liver damage markers between the study subjects (group A and B). The present study found four (4) subjects with chronic hepatitis B in the immune active phase (HBeAg positive) (table 4.6) with higher mean concentrations of urinary AFM_1 and liver damage markers as compared to those without hepatitis B envelope antigen. The four subjects with hepatitis B envelope antigen positive in relation to liver damage markers and aflatoxin exposure were not large enough to confirm the relationship between aflatoxin and hepatitis B envelope antigen. Studies involving aflatoxin and hepatitis B envelope antigen are not readily available. The findings of the present studies warrant further studies for a clear relationship of hepatitis B envelope antigen and aflatoxin exposure.

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6.0 CONCLUSION

Results from this study demonstrated significant levels of aflatoxin in hepatitis B surface antigen positive subjects as compared to hepatitis B surface antigen negative subjects. In addition, levels of aflatoxin in hepatitis C positive subjects and their control group showed no significant difference.

The study have provided for the first time in Ghana, information on aflatoxin levels in hepatitis B and C positive subjects.

6.1 LIMITATION

- Funding and time constraints limited the selection of other liver damage markers.

6.2 RECOMMENDATIONS

- The elevated levels of liver damage markers and urinary AFM_1 seen in the four subjects who tested positive for hepatitis B envelope antigen is an issue of concern. Large sample size is needed to demonstrate the relationship between aflatoxin and hepatitis B envelope antigen.
- The present studies employed subjects without liver diseases which showed a significant exposure to aflatoxin in Hepatitis B surface positive subjects. Therefore aflatoxin exposure in people with liver diseases warrants further studies especially in an aflatoxin endemic area.

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- The observations of aflatoxin exposure emphasize the need for aflatoxin exposure intervention strategies in high-risk countries; possibly targeted at postharvest stage. Therefore there is a critical need to educate people on the dangers of aflatoxin exposure to humans and to develop an economically feasible strategy to eliminate exposure to aflatoxin

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APPENDICE

PARTICIPANT INFORMATION AND CONSENT FORM

This leaflet must be given to all prospective participants to enable them know enough about the research before deciding to or not to participate.

Title of Research: Aflatoxin levels in Adults with Viral Hepatitis Infection in the Ejura Sekyedumase District of Ghana

Name(s) and affiliation(s) of researcher(s): This study is being conducted by Justice Kumi of the Department of Chemical Pathology, School of Biomedical and Allied Health Sciences, College of Health Sciences, University of Ghana, as part of **Master of Philosophy (MPhil.)** Degree in Chemical Pathology.

You have been invited to take part in a research study on exposure of aflatoxin and viral hepatitis and the risk factors associated. The researcher will first explain the study and will ask you to participate by signing this agreement which states that the study has been explained, that your questions have been answered and that you agree to participate. The researcher will explain the purpose of the study. He or she will explain how the study will be carried out and what you will be expected to do.

The researcher will also explain the possible risks and benefits of participating in the study. You should ask the researcher any questions you have about any of these things before you decide whether you wish to take part in the study. Please read the form and talk to the researcher about any questions you may have. Then, if you decide to

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participate, please sign and date this form in front of the person who explained the study to you. You will be given a copy of this form to keep.

Background: The global burden of hepatitis B is high, with about two billion people exposed worldwide and about 350 million individuals with chronic infection and at risk for complications. Acute or chronic hepatitis B viral (HBV) infection is estimated to cause 600,000 deaths each year, and about 25% of chronically infected adults later die from cirrhosis or liver cancer. About 3–4 million people are infected with hepatitis C each year, and more than 350,000 people die yearly from hepatitis C-related diseases (WHO, 2011) An estimated 170 million people worldwide are chronically infected with hepatitis C virus. The consumption of foodstuffs contaminated by aflatoxins may lead to hepatotoxic or carcinogenic effect particularly in the context of coexisting with chronic HBV infection Aflatoxin exposure may be associated with advanced liver disease in chronic hepatitis C patients in HCV-endemic regions

Purpose(s) of research: The purpose of this study is to determine the exposure of aflatoxin in Adults with viral hepatitis in Ejura Sekyedumase district and to offer them counseling about the dangers of aflatoxin poisoning and the incidence of viral hepatitis.

Procedure of the research, what shall be required of each participant and approximate total number of participants that would be involved in the research: You will be asked to provide blood sample (about 5mls). A trained biomedical laboratory scientist will insert a needle into your vein in one of your arms and draw

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some blood. This may cause pain, and discomfort at the site of needle insertion. Your blood sample will be tested for hepatitis B virus, hepatitis C virus, and liver markers (serum alanine aminotransferase, serum aspartate aminotransferase serum albumin and Gamma-glutamyl transferase). Spot urine (30mls) samples will also be taken from you into a sterile urine cup. The urine samples will used to measure aflatoxin M1. The researchers will notify you when the results are ready to share the results with you.

Risk(s): By participating in this research, you are likely to have some slight pain from the collection of blood. The procedure of blood drawing for laboratory test sample can be associated with rare risks including bruising, bleeding or skin infection. Before blood collection, your arm will be cleaned with alcohol and a new hollow needle/plastic tube will be placed in your arm to take the blood samples. When the needle goes into a vein, it hurts for a short time. The study team will try and decrease the chances of those risks/dangers happening, but if an untoward event happens, you will be immediately managed by a study physician and will be provided with free medical care in hospital.

Benefits(s): There are no direct benefits to the study participants. However, as part of the objectives of the goal, we hope that the data generated will form the firm basis to find appropriate interventions that will help reduce the risk of possible liver diseases with incidence of aflatoxins.

Confidentiality: All of your records from this study will be treated as confidential medical records. The medical results with participant's name and identifying

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information will only be available to me, the principal investigators and the study supervisors. Information collected on study forms and database will be given code numbers. No name will be recorded on the research forms or in the electronic database. The findings of this study may be reported in publications or reports but your name will not be mentioned. However, as part of my responsibility to conduct this research properly, I may allow officials from the ethics committees or the safety committee to have access to your records.

The blood and urine samples will be stored in an ice-chest and transported to the laboratory (Noguchi Memorial Institute for Medical Reseach). The remaining blood and urine samples will be destroyed three (3) years after all study analyses have been completed. All the blood and urine samples will be labeled with a code so that your identity is not revealed to the people who do the tests.

Voluntariness: You do not have to take part in this research if you do not wish to do so. Taking part in this study should be out of your own free will. You are not under obligation to do so. Research is entirely voluntary.

Alternatives to participation: This study does not involve the administration of investigational drugs or use of new curative procedures.

Withdrawal from the research: You may choose to stop participating in this research at any time that you wish to, without having to explain yourself. You may also choose not to answer any question you find uncomfortable or private.

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Consequence of Withdrawal: There will be no consequence, loss of benefit or care to you if you choose to withdraw from the study.

Contact(s): If you have any questions you may ask those now or later. If you wish to ask questions later, you may contact: Justice Kumi, School of Biomedical and Allied Health Sciences, Chemical Pathology Department, University of Ghana, on 0244876215.

I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate as a subject in this study and understand that I have the right to withdraw from the study at any time without in any way affecting my migration status in the community.

Signed by……………………. Date………………….. Place…………………………

If illiterate;

Thumbprint: ……………………..……….

In the presence of an independent literate witness:

Name…………………………………………………

Signature

(Where possible this person should be selected by the participant)

Date……………….. Place………………………………

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