Assessment of Aflatoxin Exposure among Indian Population

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Dedicated To
My mother Late Mrs. S. SARASWATHI
Whose love and blessings has made this possible

CERTIFICATE

This is to certify that the study entitled **'Assessment of aflatoxin exposure among Indian population'** has been carried out by Mrs S. Anitha, under our guidance and supervision. She has fulfilled the requirement of Nizam's Institute of Medical Sciences (University established under the state Act) regarding the prescribed period of investigation work for the award of Degree of Doctor of Philosophy. This was carried out in the Department of Medical Oncology, NIMS and in collaboration with International Crops Research Institute for the Semi-Arid Tropics (ICRISAT). The work included in this thesis is original unless stated otherwise and has not been submitted for any degree or to any university.

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DECLARATION

I hereby declare that the thesis entitled "**Assessment of Aflatoxin Exposure among Indian Populations"** submitted for the degree of Doctor of Philosophy in Department of Medical Oncology, Nizam's Institute of Medical Sciences (NIMS), Hyderabad, is a record of the bonafied research work done by me at department of Medical Oncology, Nizam's Institute Of Medical Sciences (NIMS), Panjagutta and International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru under the supervision of Prof. D.Raghunadharao, Head of the department of Medical Oncology, NIMS and co-supervision of Dr Farid Waliyar and Dr. Hari K Sudini, ICRISAT. This thesis has not formed in whole or in part, the basis for the award of any degree or diploma, earlier to this date.

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S. ANITHA

PREFACE

What impact does dietary exposure to aflatoxins has on hepatocellular carcinoma in India? Assessing the connection between aflatoxin contamination in the diets, Hepatitis B virus infection and liver cirrhosis through Hospital based study in Andhra Pradesh, India

Chronic exposure to aflatoxins was shown to cause impaired growth in children, immune suppression and liver cirrhosis, interference in micronutrient metabolism and reduced human and animal productivity. Acute severe intoxication can result in death. Most importantly, aflatoxin was shown to interact synergistically with *Hepatitis B virus* (HBV) / *Hepatitis C virus* infection. It increases the risk of hepatocellular carcinoma (HCC), one of the most common cancers in the developing countries with >400,000 new cases per annum. A joint FAO and WHO committee concluded that reduced intake of aflatoxins in HBV endemic places will have a greater impact on reducing the incidence of HCC. This requires knowledge on the level of aflatoxin exposure in populations, dietary sources of contamination and household dietary practices leading to frequent food contamination in order to identify high risk groups and to develop preventive interventions to mitigate human exposure to aflatoxins.

Aflatoxins in human body are metabolized by the liver enzymes and produces aflatoxin 8,9-epoxide, which reacts with albumin in the liver to form AFB1-albumin adducts that are major biomarkers found in peripheral blood. In order to protect human health it is important to be able to estimate aflatoxin-albumin adduct in human blood. Among many methods available for estimation immunochemical methods are simple, cost effective and adaptable to situations in developing countries. Hence this study is proposed to assess the aflatoxin exposure in Indian populations using ELISA which will lead to the development of preventive interventions to minimize the risk of liver disorders and HCC.

This thesis is divided into 9 chapters containing subsections. Chapter 1 and chapter 2 contains the introduction and review of literature. Chapter 3 contains materials and methods for the development of antibodies to detect aflatoxin-albumin adduct in human blood samples. Chapter 4 contains Results. Chapter 5 and 6 contains discussion, summary and conclusion. Chapter 7 contains references, Chapter 8 contains appendix and Chapter 9 contains Annexure I, II and Master charts.

Out line

Chapter 1	**Introduction**	**17-21**
Chapter 2	**Review of literature**	**22-51**
Chapter 3	**Materials and Methods**	**52-79**
Chapter 4	**Results**	**80-110**
Chapter 5	**Discussion**	**111-119**
Chapter 6	**Summary and conclusion**	**120-123**
Chapter 7	**References**	**124-132**
Chapter 8	**Appendix**	**133-144**
Chapter 9	**Annexures**	**145-147**

Contents

No	Content	Page No
1	**Introduction**	17-21
2	**Review of literature**	22-51
2.1	Mycotoxins	23
2.2	Overview on aflatoxins and its derivatives	25
2.3	Economic impact of aflatoxins	28
2.4	Affects of aflatoxins on health	28
2.4.1	Clinical Pathology	28
2.4.2	Ducks and Poultry	29
2.4.3	Swine	30
2.4.4	Ruminants	30
2.5	Biochemical pathway involved in metabolism of aflatoxin in human	30
2.6	Toxic affects of aflatoxins in humans and animals	33
2.6.1	Acute illness and death	33
2.6.2	Chronic affects of aflatoxin exposure	35
2.6.2.1	Cancer	35
2.6.2.2	Aflatoxin induced mutation	35
2.6.2.3	Immunologic suppression	36
2.6.2.4	Human studies	36
2.7	Overview on methods for the detection of aflatoxins	36
2.7.1	Analytical methods	37
2.7.1.1	Thin layer chromatography (TLC)	37
2.7.1.2	High pressure liquid chromatography (HPLC)	37
2.7.2	Immunological methods	37
2.7.2.1	ELISA	38
2.7.2.2	Direct competitive ELISA	38
2.7.2.3	Indirect competitive ELISA	39
2.7.2.4	Radio immuno assay (RIA)	39
2.7.2.5	Immuno affinity column assay	39
2.8	Aflatoxin detection in blood and urine	40
2.9	Management of aflatoxins in plants and human	44
3	**Materials and methods**	52-79
3.1	**Production and characterization of polyclonal antibody**	53
3.1.1	Equipment, reagents and antibodies	53
3.1.2	Preparation of AFB1-BSA adduct	53
3.1.3	Estimation of AFB1-BSA adduct concentration	54

3.1.4	Immunization of rabbit	54
3.1.5	Monitoring antibody titers	54
3.1.6	Characterization of antibody	55
3.2	**Standardization of ELISA method to detect AFB1-lysine and validation of ELISA method developed**	56
3.2.1	Preparation of standards and coating antigen	56
3.2.2	Preparation of AFB1- lysine adduct	56
3.2.3	Preparation of AFB1- ovalbumin adduct	58
3.2.4	TLC to estimate the adduct formation	58
3.2.5	Estimation of AFB1-lys and AFB1-ova adduct concentration	58
3.2.6	Evaluation of AFB1-lys adduct and AFB1-ovalbumin adduct in ELISA	59
3.2.7	IC-ELISA protocol	60
3.2.8	HPLC for validation of ELSIA method	63
3.3	**Blood sample analysis for AFB1-lysine concentration**	64
3.3.1	Equipment and reagents	64
3.3.2	Sample selection	64
3.3.2.1	Criteria of inclusion	64
3.3.2.2	Criteria of exclusion	65
3.3.3	Collection of blood sample	65
3.3.4	Isolation of albumin from serum	66
3.3.5	Estimation of protein concentration	66
3.3.6	Albumin hydrolysis with Proteinase K	66
3.3.7	SDS-PAGE for digested albumin	67
3.3.8	Artificial spiking	67
3.3.9	ELISA for the AFB1-lysine spiked and digested albumin	67
3.3.10	Testing of serum samples for AFB1-lys concentration using IC-ELISA	67
3.4	**Production of monoclonal antibody**	69
3.4.1	Equipment and reagents	69
3.4.2	Immunization	70
3.4.3	Preparation of media	70
3.4.4	Preparation of myeloma cells	71
3.4.5	Preparation of splenocytes	71
3.4.6	Counting the cells	71
3.4.7	Preparation of macrophages (Feeder cells)	72
3.4.8	fusion	72
3.4.9	Screening cells for antibody production	73
3.4.10	DAC ELISA	73
3.4.11	Multiplication of selected hybridation	73

3.4.12	Cloning of hybridomas by limiting dilution	74
3.4.13	Determination of specification of MAbs	74
3.5	**Detection of *p53* mutation using Restriction fragment length polymorphism (RFLP)**	76
3.5.1	Equipment and reagents	76
3.5.2	Sample collection	76
3.5.3	Extraction of DNA from blood sample	76
3.5.4	Agarose gel elctrophoresis	77
3.5.5	Optimation of PCR condition	78
3.5.6	Purification of PCR products	78
3.5.7	Restriction Fragment Length Polymorphism (RFLP)	79
3.5.8	Statistical analysis	79
4	**Results**	80-110
4.1	Production and characterization of polyclonal antibody	81
4.1.1	Immunization of rabbit	81
4.1.2	Monitoring antibody titer	81
4.1.3	ELISA for detection cross reaction	81
4.2	**Standardization of ELISA method to detect AFB1-lysine and validation of ELISA method developed**	83
4.2.1	Preparation of AFB1-lys and AFB1-ova adducts	83
4.2.2	Evaluation of AFB1-lys and AFB1-oval adducts	83
4.2.3	Validation of IC-ELISA using HPLC	89
4.3	**Sample analysis**	92
4.3.1	Albumin hydrolysis	92
4.3.2	Testing of recovery of AFB1-lysine in artificially spiked albumin sample	92
4.3.3	Indirect competitive ELISA for estimating the concentration of AFB1-lysine adduct in serum samples	94
4.4	**Production of monoclonal antibodies**	101
4.4.1	Immunization	101
4.4.2	Fusion and cloning	101
4.4.3	Titer and characterization of antibody	101
4.5	**Detection of *p53* Mutation using Restriction fragment length polymorphism (RFLP)**	105
4.5.1	DNA extraction	105
4.5.2	Restriction Fragment Length Polymorphism	105
5	**Discussion**	111-119
6	**Summary and conclusion**	120-123
7.	**References**	124-132
8.	**Appendix**	133-144

8.1.	Buffers and reagents for ELISA	134
8.1.1	Coating buffer	134
8.1.2	Phosphate buffer saline	134
8.1.3	Phosphate buffer saline –Tween	134
8.1.4	Substrate buffer	134
8.2	Buffer for the preparation of afaltoxin adducts and for estimation of albumin	135
8.2.1	0.01M phosphate buffer	135
8.2.2	Preparation of saturated ammonium per sulphate	135
8.3	Reagents and buffers for polyacrylamide gel electrophoresis (PAGE)	136
8.3.1	Stack gel buffer	136
8.3.2	Resolving gel buffer	136
8.3.3	Acrylamide/Bis mixture	136
8.3.4	10% ammonium per sulphate (APS)	136
8.3.5	TEMED	136
8.3.6	Electrode buffer	137
8.3.7	Plug gel composition (optional)	137
8.3.8	Resolving gel composition	137
8.3.9	Stacking gel composition	137
8.3.10	Lamelli buffer	138
8.4	Reagents for silver staining of proteins	140
8.4.1	Fixing solution	140
8.4.2	DTT wash solution	140
8.4.3	Silver nitrate solution	140
8.4.4	Developer solution	140
8.4.5	Stop solution	140
8.5	Agarose gel electrophoresis	142
8.5.1	Materials	142
8.5.2	10X elcetrophoresis buffer	142
8.5.3	Working solution	142
8.5.4	5X sample buffer	142
8.5.5	1% ethidium bromide solution solution	142
8.5.6	Working solution	142
8.5.7	procedure	143
8.6	Definition of some terms	144
9	**Annexures**	145-147
	Annexure-I	145
	Consent form, patients diet and clinical history	
	Master charts	152
	Chart 1 Aflatoxin-lysine positive status in hepatitis B positive and control samples	153
	Chart 2 *p53* mutation positive status in hepatitis B positive and control samples	169

List of Tables

Table No	Description	Page No
1	Details of some commonly occurring mycotoxins	24
2	Chemical and physical properties of aflatoxin and its derivatives	26
3	Maximum allowable limit of mycotoxins in foodstuffs, dairy products and animal feedstuffs in India 2002-2003 survey	34
4	Maximum allowable limit of aflatoxin in United States	34
5	A few options to reduce pre- and post-harvest contamination in crops	46
6	Quantitative estimation of AFB_1-lys in human serum albumin by HPLC and in HPLC fraction by IC-ELISA	90
7	Percentage recovery of AFB_1-lys from spiked human serum albumin by IC-ELISA	95
8	Effect of hydrolyzed albumin purification method on AFB_1-lys detection in IC-ELISA	96
9	Estimation of AFB_1-lys in human serum albumin by IC-ELISA	97
10	Mean afaltoxin B1-albumin according to gender, HBV infection and socioeconomic status	98
11	Concentration of AFB1-lysine pg mg^{-1} albumin (values from two experiments)	99
12	Cross reaction and minimal inhibition value of clone 1G8	102
13	Demographic Data on Patients with hepatitis B	109

List of Figures

Figure No	Description	Page No
1	Structure of aflatoxin B1 and its derivatives	27
2	Major reactions in AFB1 metabolism	32
3	AFB1-lysine conjugate	42
4	Indirect competitive ELISA for AFB1 positive and negative samples	43
5	Schematic representation of integrated management strategy for pre and postharvest management of aflatoxins in groundnut	48
6	ICRISAT intergrated approach for aflatoxin management for enhancing trade and human health.	50
7	Schematic representation of Top-down strategy (TDS) and Bottom-up strategy(BUS) for preventing human exposure to aflatoxins	51
8	Structure of AFB1-8,9-epoxide and AFB1-lys	57
9	Flow chart of indirect competitive ELISA for the estimation of AFB-lys in serum samples	61
10	Standard ELISA template for estimating the AFB1-lys in serum samples	62
11	Fusion experiment	75
12	Evaluation of polyclonal antibodies (1:40,000) against AFB1-lys, BSA and ovalbumin in IC-ELISA	82
13	Spectrophotometric graph of AFB1-lys adduct taken from 200-400 nm. This shows one peak at 275 nm and the other one at 340nm	84
14	Spectrophotometric graph of AFB1-ova from 200-400 nm shows Note two peaks, one at 275 nm and the other one at 365 nm with multiple minor peaks	85
15	TLC to evaluate AFB1-lysine adduct formation	86
16	TLC to evaluate formation of AFB1-ovalbumin adduct	87
17	Regression curve plotted using ELISA absorbance values of AFB1-lys standards measured at 405nm	88
18	Reversed phase HPLC of proteinase-K hydrolyzed human serum albumin spiked with AFB_1-lys.	91
19	Separation of purified albumin and hydrolyzed albumin in 12% SDS-PAGE and the gel was stained with silver nitrate to visualize the proteins	93
20	Growing hybridoma cells produced against AFB1-KLH conjugate	103
21	Dose response curve of aflatoxinB1-BSA.	104
22	The electrophoresis map of PCR products	107
23	The electrophoresis map of RFLP	108
24	Comparative study of *p53* mutation and aflatoxin B1 in patients and control samples	110

List of Abbreviations and Symbols

Abbreviation	Full form
AFB1	Aflatoxin B1
AFB1-lys	Aflatoxin B1-lysine
AFB1-ova	Aflatoxin B1- ovalbumin
AFB1-BSA	Aflatoxin B1- bovine serum albumin
AFB1-KLH	Aflatoxin B1-Keyhole Limpets Hemocyanin
AOAC	Association of Official Analytical Chemist
AR-ALP	Anti Rabbit – alkaline phosphatase labeled antibody
BDC	Before column derivatization
CYP450	Cytochrome P450
DAS-ELISA	Double antibody sandwich-enzyme-linked immunosorbent assay
DAC-ELISA	Direct antigen coating- enzyme linked immunosorbent assay
DCM	Dichloromethane
DNA	Deoxyribo Nucleic Acid
ELISA	Enzyme Linked Immunosorbent Assay
FAO	Food and Agriculture Organisation
GST	Glutathione-S-transferase
HBV	Hepatitis B Virus
HCV	Hepatitis C virus
HPLC	High Performance Liquid Chromatography
IARC	International Agency for Research on Cancer
ICA	Immunoaffinity column assay
ICELISA	Indirect Competitive Enzyme linked immunosorbent assay
IDH	Isocitric dehydrogenase
Mabs	Monoclonal antibodies
MCPBA	Meta- chloroperbenzoic acid
NPCL	Normal phase liquid chromatography
NPN	Non-protein nitrogen
OCT	Ornithine carbamyl transferase
PCD	Post column derivatization
PCR	Polymerase chain reaction

Pnpp	Para-Nitro Phenyl Phosphate
RAPD	Restriction fragment length polymorphism
RIA	Radio Immuno Assay
RPLC	Reverse phase liquid chromatography
SAT	Alanine transferase
TLC	Thin Layer Chromatography

List of Symbols

kg	Kilo gram
g	gram
mg	Milli gram
µg	Micro gram
ng	Nano gram
pg	Pico gram
α	Alpha
˚C	Degree Celsius
%	Percentage
M	Moles / molarity

1. INTRODUCTION

1. Introduction

Several naturally occurring toxins produced by microorganisms such as bacteria and fungi frequently contaminate food and feed, and pose a serious health risk to mammals, fish and poultry (Garcia and Park, 1998; Brown *et al.,* 1998). Of the various toxins, mycotoxins produced by the fungi are most frequent contaminants in food and feed and are responsible for several food-borne illnesses. Mycotoxins are non-volatile, low molecular weight products produced as byproducts during the primary metabolic process of the fungi (http://www.moldreporter.org/volno3/mycotoxins). The purpose of mycotoxins in fungal growth is not clearly understood, but they are believed to play a role in eliminating other microorganisms competing in the same environment and help in further invasion of the host tissues. Over 300 mycotoxins are known and they are produced by several species of fungi. Most of the mycotoxins of concern are produced by three genera: *Aspergillus, Penicillium, and Fusarium Stachybotrys* and *Myrothecium* (Bilgrami and Choudhary, 1998). However, only some species/strains of fungi are capable of producing toxins and such strains are referred as 'toxigenic strains'. Of various mycotoxins, aflatoxins produced by *Aspergillus flavus, Aspergillus parasiticus* are most important and extensive efforts have been made to understand the biosynthesis, its ill affects on humans and livestock and management.

Aspergillus flavus and *A. parasiticus* infest a number of staple crops, spices and tree nuts (eg. maize, groundnut, pistachio, cashew nut, chillies, etc) (Brown *et al.,* 1998). It is saprophytic and grows on a wide variety of substrates including decaying plant and animal debris (Payne, 1998). The fungi survive in the soil and warehouses as mycelium, conidia, and sclerotia, which resistant harsh conditions and invade crops and commodities alike. The fungi produce aflatoxins under favorable temperature, humidity and moisture content in the substrate (Sinha, 1998). The primary cause of preharvest contamination is growth of spores deposited on the crops by wind currents, insect vectors and contact. Post-harvest contamination occurs when storage conditions favor germination of spores and mold growth begins when the moisture content of the stored grains exceeds 17.5% relative humidity and temperatures exceeds 24°C. Thus mycotoxin contamination of feed grains is problematic in years with a dry growing season and wet harvest season, and such conditions are common in tropical, sub-tropical and semi-arid tropical parts of the world.

Aflatoxins were first identified in 1960s and they consists a family of toxic compounds designated as B1, B2, G1, G2 and M1 (Williams *et al*., 2004). Aflatoxin B1 is the most toxic and best studied of the compounds. The toxic effects include acute hepatitis, immunosuppression, and hepatocellular carcinoma (Williams *et al.,* 2004). In humans, the risks associated with aflatoxin consumption have been well documented, and the International Agency for Research on Cancer (IARC) has designated aflatoxin as a human liver carcinogen. For humans, aflatoxin is predominantly perceived as an agent promoting liver cancer. Moreover, aflatoxin was shown to act synergistically with Hepatitis virus B and C infections and increases the risk of liver cancer by 30 - 60 times (Groopman, 1993; Henry *et al.,* 2002). In addition, aflatoxins can cause growth inhibition and immune suppression in humans and animals. Because of the toxic effects the Food Safety regulations have been established with statutory limits of aflatoxin in food and feeds for consumption and trade (FAO, 2004). Regulations were established to monitor aflatoxins levels in food and feed samples and only those that contain zero or levels proscribed by the food safety regulations are allowed for trade and consumption (FAO, 2004). However, such procedures are poorly practices in developing countries. Thus, health hazards from consumption of aflatoxin-contaminated foods are much greater in the developing countries than the developed countries.

Although aflatoxin contamination is a global problem, it is most widespread in developing countries in the tropics. The environment, farming practices, socio-economic conditions, lack of awareness about the problem, inadequate aflatoxin monitoring skills and processing facilities makes the crops and commodities produced in these countries highly vulnerable to the contamination (Ortiz *et al*., 2005; Waliyar *et al*., 2005b). Staple foods such as groundnut, maize, spices like chillies, and several edible tree nuts (Brown *et al.,* 1998) grown in developing countries are frequently contaminated with aflatoxins and they easily make way into food chain. Most recently consumption of aflatoxin-contaminated maize has led to over 100 deaths in Kenya (Strosnider *et al.,* 2006). Mandatory analysis of food and feed products are rarely followed in developing countries to control human exposure to the aflatoxins predisposing populations to the risk of aflatoxin-linked illnesses and even cancer (Williams *et al.,* 2004). Information is limited on the individual levels of exposure to aflatoxins in Humans. Studies in West Africa showed significant proportion of

population were exposed to the aflatoxins, which provided a scope to establish intervening strategies to reduce the risk of exposure through food and also to estimate the risk of cancer (Turner *et al*., 2005).

Aflatoxin contamination in humans is analyzed through quantitative estimation of adducts of aflatoxin B1 (AFB1) that covalently binds to lysine moiety of serum albumin [AFB1-lysine (AFB1-lys)]. AFB1-lys in serum fraction serves as quantifiable biomarkers to assess the risk of exposure. Although knowledge on the metabolic cycle of AFB1 has led to the identification of several biomarkers in blood, urine, feces and tissues (Turner *et al*., 1998) that can be used to monitor the AFB1 exposure, but the most well studied biomarker has been the AFB1-lys. It has been shown that AFB1-lys concentration in albumin reflects the level of DNA damage in the liver, which is the target organ for liver cancer (Skipper and Tannenbaum, 1990). Moreover, AFB1-lys in the blood can be estimated up to 20 days or even a month following its formation. Most importantly, antibodies can be produced to AFB1-lys that can be conveniently used to estimate the AFB1-lys adducts in albumin fraction by enzyme-linked immunosorbent assay (ELISA).

ELISA is by far the most widely used serological test for the detection of aflatoxins, because of its simplicity, adaptability and sensitivity (Wilson *et al.,* 1998). This is based on the reaction between the antigen and antibody that is produced against. Immunological recognition is based on the special complement of specific groups of the antigen with those of the antibody. Immuno-specificity is recognized through the action of the associated enzyme label on a suitable substrate. For the detection of AFB1-lys, competitive ELISA is used (Waliyar *et al.,* 2005a). In this a known amount of AFB1-ovalbumin is adsorbed to the plate surface. Competition is between antibody with the toxin present in the albumin sample or in the standard. The antibodies that react with AFB1-lys in the sample get neutralized and eliminated. Whereas the unbound antibody will bind to the AFB1-ovalbumin coated to the plate. The bound antibodies were detected using enzyme labeled anti-species antibody, which provides the measurement of the reaction.

This study was planned to standardize the competitive ELISA method for the estimation of AFB1-lys in human serum albumin. Recently, polyclonal antibodies for

AFB1-lys were produced at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT). These antibodies were used to standardize the method and the efficacy of these antibodies was validated by comparing with the antibodies produced at Columbian University, USA (kindly provided by Dr Regina M. Santella, Professor of Environmental Health Sciences, Mailman School of Public Health, Columbia University). This method would be useful for assaying the level of aflatoxin exposure among Indian population.

Objectives:

The objectives of this study are:

- To produce, standardize and validate the polyclonal and monoclonal antibody against afaltoxin albumin adduct.
- To standardize competitive ELISA method for estimating the AFB1-lys in serum fraction
- To do transactional studies to determine aflatoxin exposure by measuring aflatoxin biomarker among various liver diseased patients in Hyderabad.
- To standardize and detect aflatoxin-albumin adduct from Hepatitis B positive samples and to detect 249^{ser} mutation of p*53* gene using PCR/RFLP.

2. REVIEW OF LITERATURE

2. Review of Literature

2.1. Mycotoxins

Many agricultural commodities are vulnerable to attack by a group of fungi that are able to produce toxic metabolites called mycotoxins. Mycotoxin contamination in crops and commodities are regarded as unavoidable and has been a serious challenge to food safety.

Mycotoxins are toxic secondary metabolites of low molecular weight produced by certain species of fungi (Chu, 1992). They are usually named on the basis of the fungus that produces them. For example, aflatoxin produced by *Aspergillus flavus* uses A for *Aspergillus* and fla for species *flavus* along with the word toxin as suffix. There are three major genera of fungi that produce mycotoxins: *Aspergillus, Fusarium* and *Penicilium*. Mycotoxin producing fungi grow on a wide spectrum of crops, food and feed and invade at any time during pre-harvest, post-harvest, processing, transport, storage and distribution. The growth of storage fungi, and their production of secondary metabolites, presents a major problem to agriculture, feed and food industries worldwide.

Fungal invasion of stored agricultural products leads to quality loss in many ways: unacceptable physicochemical changes in the products such as deterioration of color, texture and taste, development of fungal odors (Abramson, 1991; Kaminski and Wasowicz, 1991), reduction of seed germination, energy and nutritional loss, production of allergens affecting livestock (Flannigan *et al*., 1991) and formation of toxic metabolic products such as mycotoxins. The Food and Agriculture Organization (FAO) of the United Nations has reported every year, at least 25% of the world's food crops are contaminated with mycotoxins.

The mycotoxin producing fungi are aerobic and can be both pathogenic to plants and/or saprophytic with them. Common members of mycotoxin family include: aflatoxin, trichothecenes, zearalenone, fumonisin, ochratoxins and slaframine (Table 1). The main toxigenic species are *A. flavus* and *A. parasiticus,* which produce aflatoxins and *A. ochraceus* for ochratoxins (Abramson, 1998).

Table 1. Details of some commonly occurring mycotoxins (Chu, 1995)

Mycotoxins	Major producing fungi	Typical substrate in nature	Biological effect
Alternaria mycotoxin (AM)	*Alternaria alternata*	Cereal grains, tomato, animal feeds	M, Hm
Aflatoxin B1 and other aflatoxins	*Aspergillus flavus, Aspergillus parasiticus*	Peanuts, corn, cotton seed, cereals, figs, most tree nits, milk, sorghum, and walnuts.	H,C,M,T
Citrinin	*Penicillium citrinum*	Barley, corn, rice, walnuts.	Nh, C, M
Cyclopiazonic acid	*Asprgillus flavus, P. cyclopium*	Peanuts, corn, cheese	Nr, Cv
Deoxynivalenon	*Fusarium graminarum*	Wheat, corn	Nr
Cyclochlorotine	*P. islandicum*	Rice	H,C
Fumonisins	*F. moniliforme*	Corn, sorghum	H, Nr, C(?),R
Luteoskyrin	*P. islandicum, P. ruguulosum*	Rice, sorghum	H, C, M
Monoliformin	*F. monoliforme*	Corn	Nr, Cv
Ochratoxin A	*Aspergillus ochraceus P. verrucosum*	Barley, beans, cereals, coffee, feeds, maize, oats, rice, rye, wheat	Nh, T
Patulin	*P. patulum, P. urticae, Aspergillus clavatus*	Apple, apple juice, beans, wheat	Nr, C(?), M
Penicillic acid	*P. puberulum, Aspergillus ochraceus*	Barley, corn	Nr, C (?), M
Penitrem A	*P. paitans*	Feedstuffs, corn	Nr
Roquefortine	*P. roqueforti*	cheese	Nr
Rubratoxin B	*P. rubrum, P. purpurogenum*	Corn, soybeans	H, T
Sterigmatocystin	*Aspergillus versicolor, Aspergillus nidulans*	Corn, grains, cheese	H, C, M
T-2 Toxin	*F. sporotrichioides*	Corn, feeds, hay	D, ATA, T
12-13	*F. nivale*	Corn, feeds, hay, peanuts, rice	D, Nr
Epoxitrichothecenes other than T-2 and DON Zearalenone	*F. grminearum*	Cereals, corn, feeds, rice	G.M

Cv = cardiovascular lesion; D = dermatoxin; G = genitotoxin and estrogenic effects; H = hepatotoxic; Hr = hemorrhagic; M = Mutagenic; Nr = neurotoxins; R = respiratory; T = teratogenic; C = carcinogenic; C(?) = Possible carcinogenic effect; ATA = Alimentary toxin aleukia; *P.* = *Penicillium; F* = *fusarium*.

Of the various mycotoxins, aflatoxins are most widespread and high priority is given to its regulation due to carcinogenic, immunosuppressive and teratogenic nature. Although several *Aspergillus* species produce aflatoxins, *A. flavus* is the most commonly occurring species and it is highly aggressive.

2.2. Overview of aflatoxins and their derivatives

Aflatoxins are a group of closely related secondary metabolites of the fungi *A. flavus* and *A. parasiticus*. *A. flavus* produces aflatoxin B1 (AFB1) and aflatoxin B2 whereas *A. Parasiticus* produces aflatoxin B1, B2, G1 and G2. Of these four, AFB1 is most potent toxin (Payne, 1998; Abramson, 1998). AFB1 is produced by *A. flavus, A. parasiticus, A. nomius, A. bombycis, A. ochraceoroseus, A. pseudotamarii* and *Emericella venezuelans,* on a wide range of tropical and subtropical agricultural commodities (http://www.anci.cornell.edu/plants/toxicagents/mycotoxin.html). Of the various species, *A. flavus* is most widespread and common. It is a saprophyte during most of its life cycle and grows on a variety of substrates including decaying plant and animal debris. The major factors that influence soil populations are temperature and soil moisture. *A. flavus* can grow at temperatures from 12-48 ºC and at water potentials as low as –35 Mpa (Klich *et al.,* 1994). The optimum temperature for growth is 25 to 42 ºC. The fungi most commonly occur on groundnut (*Arachis hypogaea*), maize (*Zea mays*), several tree nuts (pistachio, cashew nuts, Brazil nuts, etc.), figs (*Ficus carica*), and on many other crops. Contamination mostly occurs on post-harvest products, stored at high temperatures and high humidity, but also known to occur in the fields before harvest on crops subjected to drought stress (Payne and Brown, 1998; Waliyar *et al.,* 2003).

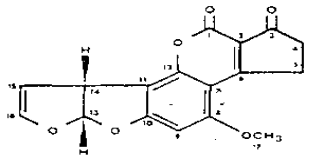
Aflatoxins are basically difuranocoumarin compounds, which include aflatoxin B1, B2, B2a, G1, GM1, G2, G2a, M1, M2, GM2, P1, Q1, R0, RB1, RB2, AFL, AFLH, AFLM, and methoxy, ethoxy and aceto derivatives (Bilgramy and Choudhary, 1998). They are closely related compounds with small differences in chemical composition (structures of few aflatoxins given in Fig. 1). Several mycotoxins exert biological actions that impair the effectiveness of native defense mechanisms and immunity (Pier *et al*., 1979). AFB1 ($C_{17}H_{12}O_6$) is the naturally occurring and most prevalent form and also the most potent of these toxins. It is a hepatotoxin, a hepatic carcinogen and mutagen. Toxin response depends on how the molecule is metabolized in the liver. It is

commonly associated with human liver cancer in association with *Hepatitis B virus* (HBV) infection (Kirk *et al.,* 2005). The chemical and physical properties of aflatoxins and its derivatives are given in Table 2.

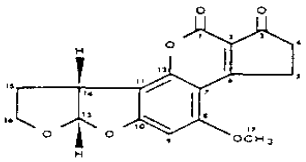
Table 2. Chemical and physical properties of aflatoxin and its derivatives (Bilgramy and Choudhary, 1998)

Aflatoxin	Molecular formula	Molecular Weight	Melting Point
B1	$C_{17}H_{12}0_6$	312	268-269
B2	$C_{17}H_{14}0_6$	314	286-289
G1	$C_{17}H_{12}0_7$	328	244-246
G2	$C_{17}H_{14}0_7$	330	237-240
M1	$C_{17}H_{12}0_7$	328	299
M2	$C_{17}H_{14}0_7$	330	293
B2A	$C_{17}H_{14}0_7$	330	240
G2A	$C_{17}H_{14}0_8$	346	190

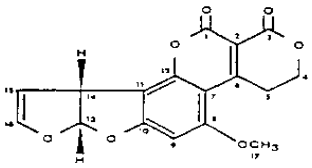
Figure 1. Structure of aflatoxin B1 and its derivatives (Sabioni and Sepai, 1998)



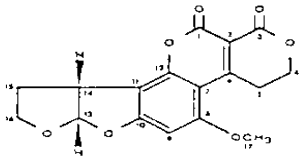
(a) Aflatoxin B_1



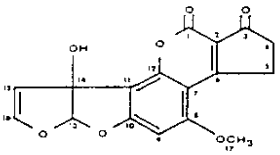
(b) Aflatoxin B_2



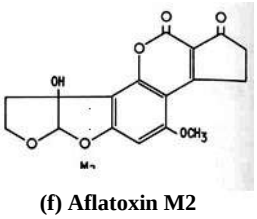
(c) Aflatoxin G_1



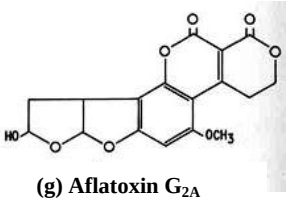
(d) Aflatoxin G_2



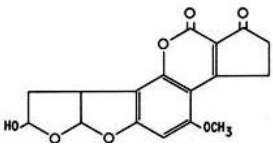
(e) Aflatoxin M_1



(f) Aflatoxin M2



(g) Aflatoxin G_{2A}



(h) Aflatoxin B_{2A}

2.3. Economic impact of aflatoxins

The economic impact of aflatoxins derives directly from crop and livestock losses, human health as well as indirectly from the cost of regulatory programs designed to reduce risks to animal and human health. As per the FAO estimates, about 25% of the world's food crops are affected by mycotoxins, of which the most dangerous is aflatoxins (Garcia and Park, 1998). Aflatoxin losses to livestock and poultry producers from aflatoxin-contaminated feeds include death and the more subtle effects of immune system suppression, reduced growth rates and losses in feed efficiency. Other adverse economic effects of aflatoxins include lowered market potential of food and fiber crops.

2.4. Affects of aflatoxins on health

The historic discovery of aflatoxins was due to the death of turkeys in the United Kingdom as a result of consuming contaminated groundnut meal imported from Brazil (Bhat *et al*., 1978). The harmful effects of consuming contaminated groundnut cake have been mainly observed in poultry and milch cattle (Bhat *et al*., 1978). The effect of poultry includes mortality, feed refusal, slowed growth, fertility and reproduction problems, and decreased resistance to diseases. Cattle fed with aflatoxin-contaminated feed result in secretion of metabolites of aflatoxin (AFM1) in the milk.

Early signs of aflatoxicosis in livestock are reduction in feed intake and weight loss, often followed by rapid death. The susceptibility of the animals depends on the species (ducklings, rabbit, turkey, chicken, swine, cattle, and sheep in decreasing order), the form of aflatoxin and on the animals' nutritional status. The liver is the primary target organ in most cases.

2.4.1. Clinical pathology: The clinico-pathologic features of aflatoxins depend on hepatic injury. It is dose dependent. Doses adequate to produce death within 24 hours will result in detectable liver damage within 3 hours and several alterations in liver function within 6 hours. Marked elevation in the serum alanine transferase (SAT), ornithine carbamyl transferase, and isocitrate dehydrogenase (IDH) were reported. Reduction in the serum levels of albumin, albumin-globulin ratio, non-protein nitrogen (NPN), and urea were also reported (Edds, 1979).

A constant response to AFB1 is the proliferation of small bile ductules. This is usually accompanied by loss of hepatic glycogen, fatty infiltration, fibroblastic proliferation, and perivascular edema (Nelson, 1979). These degenerative changes lead to necrosis that is usually localized in one part of the lobule (centrolobular or midzonal). Edema of the gallbladder has frequently been noted in pigs (Nelson, 1979).

Carcinogens at the cellular level cause degranulation of the endoplasmic membranes. Hepatic changes at the cellular level in aflatoxin-treated calves were: loss of ribosomes from the endoplasmic reticulum, loss of nuclear chromatin material and altered nuclear shapes (Lynch *et al*., 1971). For example in cattle, AFB1 is metabolized by hepatic microsomal mixed function oxidase. The hydroxylation of AFB1 to AFM1 during lactation is of particular concern. AFM1 is also found in the liver, kidney and urine of sheep (Masri *et al*., 1974)

Aflatoxicosis in farm animals produces growth retardation, fatty liver and a decrease in the lipid content of extra hepatic tissues. The accumulation of fat in liver has been suggested to be due to a blockade to fat removal from the liver. The aflatoxin effect on lipid metabolism is also marked by reduction in such fat-soluble vitamins as vitamin A (Lynch, 1979).

However, the major effect of aflatoxin is on the cell-mediated immune system. This failure has been demonstrated in poultry as fowl cholera and in swine as erysipelas. Aflatoxin consumption has also been reported to increase susceptibility of poultry to salmonellosis, candidosis and coccidiosis and of calves to fascioliasis (Pier *et al*., 1979). The diminished resistance and immunity are thus a problem of substantial economic importance.

2.4.2. Ducks and poultry: Ducklings are the most susceptible class of livestock to aflatoxicosis and are preferentially used in bioassays for aflatoxins in feeds. Ducklings show a decreased utilization of dietary protein on diets containing even 70 μg kg^{-1} AFB1. Whereas chickens show lowered levels of performance on diets containing >250 μg kg^{-1} AFB1, at higher levels (>500 μg kg^{-1}), liver lesions become severe.

2.4.3. Swine: Relatively low concentration of aflatoxin B1 reduces average daily gain and feed efficiency in piglets fed on contaminated maize, but this affect was reversed by increasing the concentration of crude protein in the diet and by the addition of fat. It was shown that aflatoxin levels of 100 to 300 μg kg^{-1} in swine rations usually do not produce toxin effects from weaning to market, but, levels greater than 400 μg kg^{-1} may produce aflatoxicosis (Edds, 1979). Residues of AFB1 and AFM1 may be present in liver, kidney and muscle tissues in swine fed on diets containing only 100 μg kg^{-1} aflatoxin B1 and these residues may be hazardous for human consumption (Edds, 1979).

2.4.4. Ruminants: Rumen fermentation does not appear to detoxify aflatoxins, but aflatoxins may inhibit rumen microorganisms. Only 0.7% to 1.4% of an oral dose of AFB1 was excreted in milk from lactating goats, 25% of the aflatoxin in this milk was M1. About 50% of the oral dose was detected in feces (Helferich *et al*., 1986).

2.5. Biochemical pathway involved in metabolism of aflatoxin in human

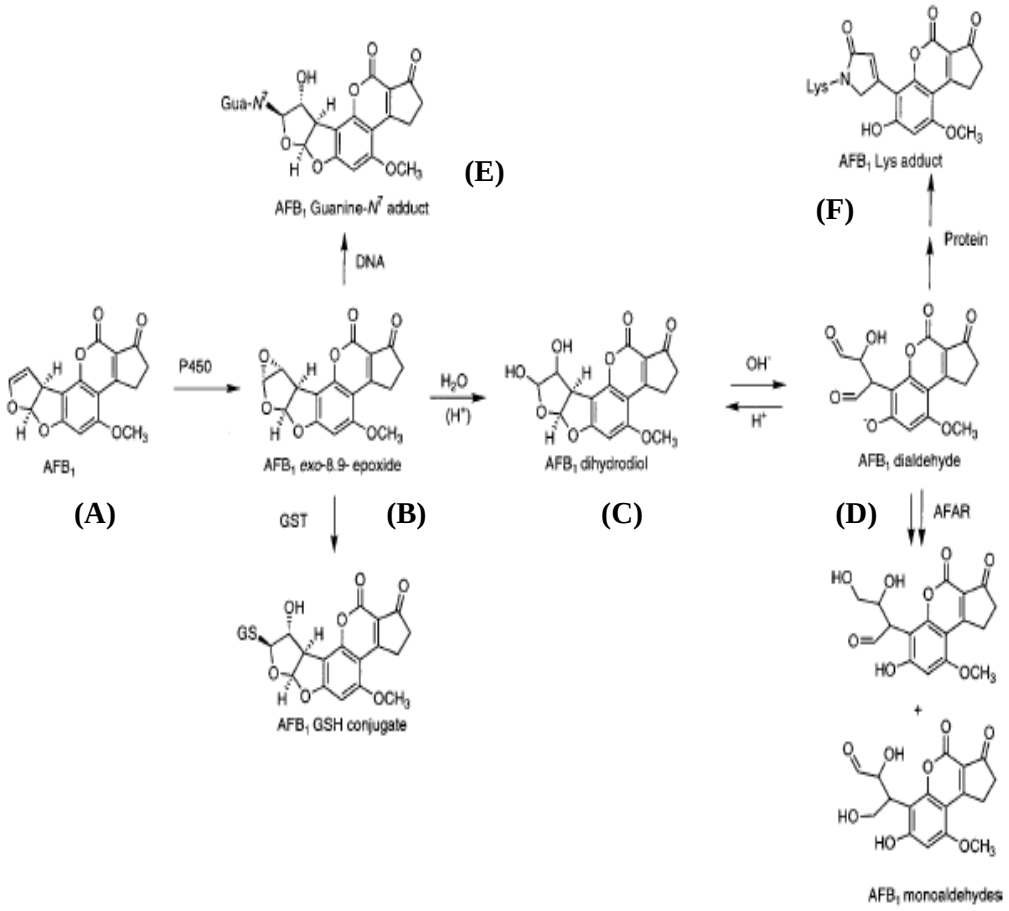
Aflatoxins are activated by several cytochrome P-450s, including CYP2A3, CYP2A6 and CYP3A4 (Wojnowski *et al*., 2004). AFB1 and G1 (Fig. 1a, and c) have an olefinic double bond at the 8,9-position, and they are more mutagenic and carcinogenic than aflatoxin B2 and G2 (Fig. 1b,d), which are saturated and have an ethylenic bond at this position. This implies that the olefinic 8,9-bond is the site of activation.

AFB1 (Fig. 1a) is metabolized to AFB1 8,9-eopoxide, which can bind to protein and DNA, predominantly at the N7 position of guanine. AFB1 reacts with hepatic macromolecules and with serum albumin in a dose dependent manner. The important step in the process of carcinogenesis is the formation of AFB1-epoxide. AFB1 is activated by cytochrome P450 to AFB1 8,9-*exo* epoxide (Fig. 2B) and AFB1 8,9-*endo* epoxide. But it is the *exo* epoxide which binds to form the predominant AFB1- N7 guanine adducts (Fig. 2E). The epoxide ring is positioned above the plane and in *trans* to the 5a and 9a protons in the *endo* epoxide, hindering the reaction. But in the *exo* epoxide, the epoxide ring is positioned below the plane and in *cis* to the 5a and 9a protons assisting the reaction (Wild and Turner, 2002).

AFB1 itself is a relatively innocuous molecule but cytochrome P450 enzyme and some other oxygenases (to a lesser extent) oxidize it to the 8,9 epoxide, which has a central role in all succeeding reactions (Guengerich *et al.,* 2002). 8,9-*exo* epoxide hydrolyzes rapidly to dihydrodiol (Fig. 2C) that in turn undergoes slow, base-catalyzed ring opening to a dialdehyde phenolate ion. AFB1 dialdehyde does not bind to DNA but forms Schiffs base with primary amine groups, e.g. lysine, to form protein adducts such as aflatoxin albumin (Fig. 2F).

The formation of AFB1-glutathione adduct is an important detoxification pathway. This process is catalyzed by species-specific glutathione-S-transferase (GST). It appears that animals that are very susceptible to AFB1 lack an efficient GST. One percent to two percent of the administered dose is excreted into urine as the glutathione (Raj and Lotlikar, 1984).

Figure 2: Major reactions in AFB1 metabolism (Figure source, Guengerich *et al.*, 2002)



2.6. Toxic affects of aflatoxins in humans and animals

Aflatoxicosis is the poisoning that results from ingesting aflatoxins. Two forms of aflatoxicosis have been identified

- Acute severe intoxication, which results in direct liver damage and subsequent illness or death.
- Chronic sub symptomatic exposure (leads to cancer, immuno suppression, etc.).

2.6.1. Acute illness and death

High levels of aflatoxin exposure produces an acute necrosis, cirrhosis and carcinoma of liver exhibited by hemorrhage, acute liver damage and edema, alteration in digestion and absorption and metabolism of nutrients (Williams *et al.,* 2004). No animal species is immune to the acute toxic effects of aflatoxins including humans. However, humans have an extraordinarily high tolerance for aflatoxin exposure and rarely succumb to acute aflatoxicosis.

Animals were found to differ in the median lethal dose for AFB1. Susceptible species such as rabbits and ducks have a low (0.3 mg kg^{-1}) median lethal dose, whereas chickens (18 mg kg^{-1}) and rats have greater tolerance. Adult human usually have a high tolerance of aflatoxin and in the reported acute poisoning, it is usually the children who are most vulnerable to aflatoxin-related deaths. Economic pressures have created a double standard for allowable contamination of commodities destined for human and animal consumption. Human foods are allowed 4-30 ppb aflatoxin, depending on the country involved (Henry *et al*., 1999). The maximum allowable limits of mycotoxins in India and in US were given in Table 3 and Table 4 respectively.

Table 3. Maximum allowable limit of mycotoxins in foodstuffs, dairy products and animal feedstuffs in India 2002-2003 survey (FAO, 2004)

Commodity	Mycotoxins	Limits (µg kg^{-1})
Food: all food products	AFB1, B2, G1, G2 and M1	30
Feed: Peanut meal (export)	AFB1	120

Table 4. Maximum allowable limit of aflatoxin in United States (FAO, 2004)

Aflatoxin Concentration PPB (Parts per billion)	Affects on animal Health
20	Highest level allowed for humans
50	Highest level allowed for animals
100	Slowed growth of young ones
200-400	Slowed growth of adults
>400	Liver damage and Cancer

2.6.2. Chronic affects of aflatoxin exposure

Chronic sub-clinical exposure does not lead to as dramatic symptoms as acute aflatoxicosis. Children however are particularly affected by aflatoxin exposure, which leads to stunted growth and delayed development. Chronic aflatoxin exposure has major effects on nutritional status in animals, but, as with immunotoxins, thresholds for these effects were not defined for any species (Roebuck and Maxuitenko, 1994). Chronic exposure also leads to a high risk of developing liver cancer, as the metabolite AFB1 can intercalate into DNA and alkalate the bases through its epoxide moiety (Roebuck and Maxuitenko, 1994).

2.6.2.1. Cancer: Hepatocellular carcinoma (HCC) is the sixth most common cancer, accounting for about 5% of all human cancers and the second cause of cancer death in the world (Behnoush *et al.,* 2011). For humans, aflatoxin is predominantly perceived as an agent promoting liver cancers. The International Agency for Research on Cancer (IARC) classified AFB1 as a Class 1 carcinogen. A strong synergy has been shown between aflatoxin and Hepatitis B virus (HBV) and C virus (HCV). In HBV infected persons, aflatoxin is 30 times more potent than in persons without the virus. The suggested mechanism for the synergy is that aflatoxin suppresses DNA repair mechanism that helps limit the development of cancer from HBV (Williams *et al.,* 2004). A factor in this greater potency of aflatoxin in HBV positive people is that HBV reduces the person's ability to detoxify aflatoxin. In some areas where aflatoxin contamination and HBV occur together, hepatomas are the predominant cancer and they may be a major cause of death (Henry *et al*., 2002).

2.6.2.2. Aflatoxin induced mutation:

Epidemiological studies in high aflatoxin contaminated areas indicate that dietary AFB1 contributes to the development of HCC and the two main risk factors, HBV and AFB1, have a synergistic effect in liver carcinogenesis (Jiang *et al.,* 2005). The molecular hallmark of AFB1 intoxication in relation to HCC is a specific mutation at codon 249 of the *TP53* gene. This mutation is a single-base substitution at the third base of codon 249 (AGG to AGT), which replaces an arginine "R" by a serine "S". This mutation has been reported in about 75% of HCC cases in high incidence areas (China or East Africa). Such a mutation is not detected in HCC cases from non-aflatoxin contaminated areas (Montesano *et al.,* 1997).

2.6.2.3. Immunologic suppression: In animal experiments, AFB1 has been shown to induce thymic aplasia, reduce T-lymphocyte function and number, suppress phagocytic activity and reduce complement activity. Many studies conducted in poultry, pigs, and rats showed that exposure to aflatoxin results in suppression of the cell-mediated immune response. (Pier, 1986; Reddy *et al*., 1987; Richard *et al*., 1978).

Macrophages play a major role in host defense against infection. They present antigen to lymphocytes during the development of specific immunity. Macrophages also increase their phagocytic activity and release various active products, such as cytokines and reactive intermediates, to carry out nonspecific immune responses. Several reports suggest that aflatoxin impairs the function of macrophages in animal species (Neldon-Ortiz, 1991).

2.6.2.4. Human studies: Jiang *et al*. (2005) studied the aflatoxin levels and cellular immune status in Ghanaians. They examined the cellular immune status in relation to levels of AFB1 –albumin adducts in plasma. The percentage of leukocyte immunophenotypes in peripheral blood, CD4+ T cell proliferative response, CD4+ Th and CD8+ T cell cytokine profiles and monocyte phagocytic activity were measured using flow-cytometry. They showed that participants with high AFB1 level had significantly lower percentages of CD3+ and CD19+ cells. Also, the percentage of CD8+ T cells that contained perforin or both perforin and granzyme A were significantly lower in participants with high AFB1 levels when compared with low AFB1 level (Jiang *et al*., 2005).

2.7.Overview on methods for the detection of aflatoxins

Ensuring the safety and wholesomeness of foods would be impractical without reliable methods of laboratory analysis to determine whether products are up to specified standard (Wood and Trucksess, 1998). Many methods are available for the estimation of aflatoxins. However, it is important to choose the method that gives the most reliable results for the commodity being analyzed. The traditional method for analysis includes Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC). The basic steps in each method include extraction, liquid partition, purification, separation and quantification. Recently, several simple but sensitive ELISA method have been developed, evaluated and approved for qualitative

screening and quantification of aflatoxins (Trucksess and Wood, 1994; Anitha *et al.,* 2011)).

2.7.1. Analytical methods

2.7.1.1 Thin layer chromatography (TLC), also known as flat bed chromatography or planner chromatography, is one of the widely used methods in aflatoxin analysis. Since 1990, it has been considered the official method of the Association of Official Analytical Chemist (AOAC) and the method of choice to identify and estimate aflatoxins as low as 1 ng g^{-1}. TLC needs high sample purification. The major disadvantage is that the procedure is inherently variable, so the coefficient of variation is large and the precision poor (Wilson *et al*., 1998).

2.7.1.2. High Performance Liquid Chromatography (HPLC) gives an accurate quantification of the aflatoxin from the various samples, including blood. But, the success of HPLC separation for mycotoxin analysis depends on factors such as extraction, cleanup, concentration, separation, detection techniques and HPLC conditions suitable for the specific compounds and matrices of concern (Wilson *et al.,* 1998), which makes this technique difficult. Liquid chromatography requires analytic application, stationary phase and mobile phase. Liquid chromatography methods for the detection of aflatoxins in foods include normal phase LC (NPCL), reverse phase LC (RPLC), with pre or before column derivatization (BCD), RPLC followed by post column derivatization (PCD), and RPLC with electrochemical detection (Wilson *et al.,* 1998).

Usually, in analytical techniques like TLC and HPLC, the extracted sample needs clean up before analysis, to remove co-extracted materials that often interfere with the determination of the target. Both the TLC and HPLC are laborious and time consuming.

2.7.2. Immunological methods

Immunological methods using polyclonal or monoclonal antibodies have become the cornerstone of rapid testing of foods and feeds for aflatoxins and other mycotoxins (Wilson *et al.,* 1998). Immunological recognition is based on the special complement of specific groups of the antigen with those of the antibody. Since mycotoxin

molecules (including AFB1) cannot elicit immune response, the molecules are conjugated to carrier proteins for immunization purpose (Chu, 1994). For instance, polyclonal antibodies are produced against AFB1 by immunizing animals with a AFB1-bovine serum albumin (BSA) conjugate (Waliyar *et al.,* 2005). For aflatoxin detection in blood, polyclonal antibodies have to be raised against the aflatoxin-lysine derivative, which is conjugated to carrier protein like BSA. Similar immunization procedure is followed even for the production of monoclonal antibodies. The monoclonal antibodies are generally raised by the fusion of immunized mice spleen cells with a myeloma cell line (Chu, 1994). Various serological methods such as ELISA, Radio Immuno Assay (RIA), immunoaffinity column assay (ICA) and lateral flow assays have been developed for the estimation of aflatoxins in various crops, commodities, milk and even in blood (Chu, 1990).

2.7.2.1. ELISA: It is a highly sensitive, simple and cost-effective technique. It is based on an antigen antibody reaction. It gives accurate quantification of aflatoxin in various commodities, including estimation of aflatoxin exposure in blood by detecting AFB1-lys. Sample clean-up is not necessary after extraction. The results are reproducible and comparable with HPLC. Because of all these advantages, many researchers are now concentrating on this technique.

Two types of ELISA have been used in the analysis of aflatoxins: (i) Indirect ELISA (Fig 4); and (ii) Direct ELISA. Both forms are heterogenous competitive assays, which involve the separation of free (untreated) toxin in liquid phase from the bound toxin in solid-phase.

2.7.2.2. Direct Competitive ELISA: In the typical, direct ELISA, the binding of aflatoxin-enzyme conjugate by the immobilized antibodies is inhibited by the presence of aflatoxin in the test sample. The bound enzyme catalyzes the transformation of the substrate to a coloured complex. The colour intensity formed is inversely related to aflatoxin concentration. Alkaline phosphatase and horse radish peroxidase catalyze the oxidation of the substrates, para- nitrophenyl phosphate and tetramethylbezidine, respectively, to form a yellow and blue coloured complexes, respectively (Wilson *et al*., 1998). ELISA plates, beads, membranes (cup, card, probe test) have been used as

solid support for ELISA. This is technically more demanding and requires conjugation of enzyme label directly to the anti-aflatoxin antibodies.

2.7.2.3. Indirect competitive ELISA: In the indirect ELISA the binding of primary antibody to the immobilized antigen is inhibited by the presence of aflatoxin in the test sample. The secondary antibody that is conjugated to the enzyme can find the primary antibody and the addition of substrate is catalyzed by the enzyme conjugate. This produces colored product. The intensity of the colour developed is inversely related to aflatoxin concentration (Fig. 4).

2.7.2.4. Radio immno assay (RIA): The principle is similar to that of ELISA. But, radiolabeled aflatoxin is used in RIA. In competitive RIA, the unlabelled aflatoxin standard or the aflatoxins in the test solution and the labeled aflatoxin in the assay system are competing for the limited number of binding sites on the antibodies (Chu, 1990). The amount of aflatoxins in a sample is inversely related to the amount of radiolabeled aflatoxin in the solution. Only a small amount of antibody is required for RIA. However, the radioisotopes used in the assays present disposal problems. Therefore, the use of non-isotopic labels such as enzymes has gradually replaced the use of RIA.

2.7.2.5. Immuno affinity column assay: Affinity columns are prepared by adsorption of the antibodies onto a gel material contained in a small plastic cartridge. Aflatoxins are captured from test solutions by the immunospecific antibodies (Trucksess *et al*., 1991). After non-binding impurities have been washed from the cartridge, aflatoxins are desorbed with methanol, then estimated by solution fluorometry or transferred to the liquid chromatographic reversed-phase column for further separation and fluorescence determination (Chu, 1992). The use of affinity columns to capture and concentrate aflatoxins has several advantages: increased selectivity, the ability to trap the aflatoxins in large volumes of test samples (biological fluids), and integration with other analytical techniques. However, large amounts of antibodies are needed to prepare the immunosorbent columns and the cost is greater than RIA and ELISA.

2.8. Aflatoxin detection in blood and urine

Epidemiological studies in the areas of high incidence of hepatoma throughout Africa and Southeast Asia have suggested that the exposure to aflatoxins may play a role in primary liver cancer (Rose *et al.,* 1992). It has not been clarified whether aflatoxin alone or an interaction with HBV infection cause the hepatomas (Rose *et al.,* 1992). This has necessitated the need for methods to determine the human exposure to aflatoxins, to assess the risk of liver cancer and other aflatoxin-related illnesses and, to demonstrate the relationship between exposure to aflatoxins and the etiology of disease. Now, various methods have been developed to measure aflatoxin metabolites in milk, sera and urine (Wilson *et al.,* 1998).

Accurate and sensitive biological markers of carcinogen (aflatoxin) exposure are now used to estimate cancer risk. Biomarkers can be metabolites, DNA adducts or protein adducts. Knowledge of the metabolic fate of AFB1 has led to the use of several biomarkers in blood, urine, feces and tissues that can be used to monitor AFB1 exposure. AFB1 is among the few compounds for which the relationship between biomarkers and exposure has been extensively studied (Turner *et al*., 1998).

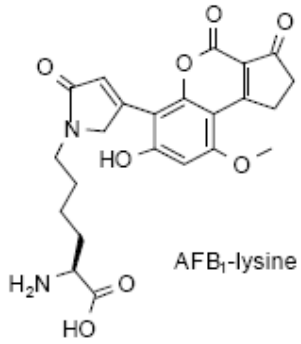
There are two techniques that have been used most often to detect levels of aflatoxin in humans. The first method is measuring the DNA adducts, AFB1-guanine, in the urine of subjects. Aflatoxin once ingested is metabolized by the cytochrome P450 system, primarily in the liver. The resulting reactive AFB1-8,9-epoxide binds to DNA (Essigman *et al.,* 1977; Martin and Garner, 1977), forming AFB1-guanine adduct. Presence of these breakdown products indicates exposure to aflatoxin in the past 24 hours. However, this technique has a significant flaw in that it only produces a positive result in approximately one third of positive test subjects. Additionally, due to the half-life of this metabolite, the level of AFB1-guanine measured can vary significantly from day to day, based on diet. Thus, is not useful for assessing long term exposure. The persistence of AFB1-formamidopyrimidine DNA adduct may be a useful biomarker of AFB1 exposure but is limited by availability of tissue.

Another technique is the measurement of the AFB1-albumin adducts level in the blood serum. AFB1-8,9-epoxide formation also leads to AFB1-protein binding via AFB1-8,9-dihydrodiol (Sabbioni *et al.,* 1987). This binding occurs specifically to the

amino acid, lysine, of albumin. Because human albumin has a half-life of approximately 20 days, AFB1-albumin adduct detection provides a useful biomarker of cumulative AFB1 exposure. Also, it is less invasive to collect blood samples for analysis than to surgically remove tissue samples from individual. This approach is significantly more accurate, as positive results are generated in 90% of positive test subjects (Turner et al., 1998). This test is also useful for measuring long-term exposure, as it remains positive for two to three months.

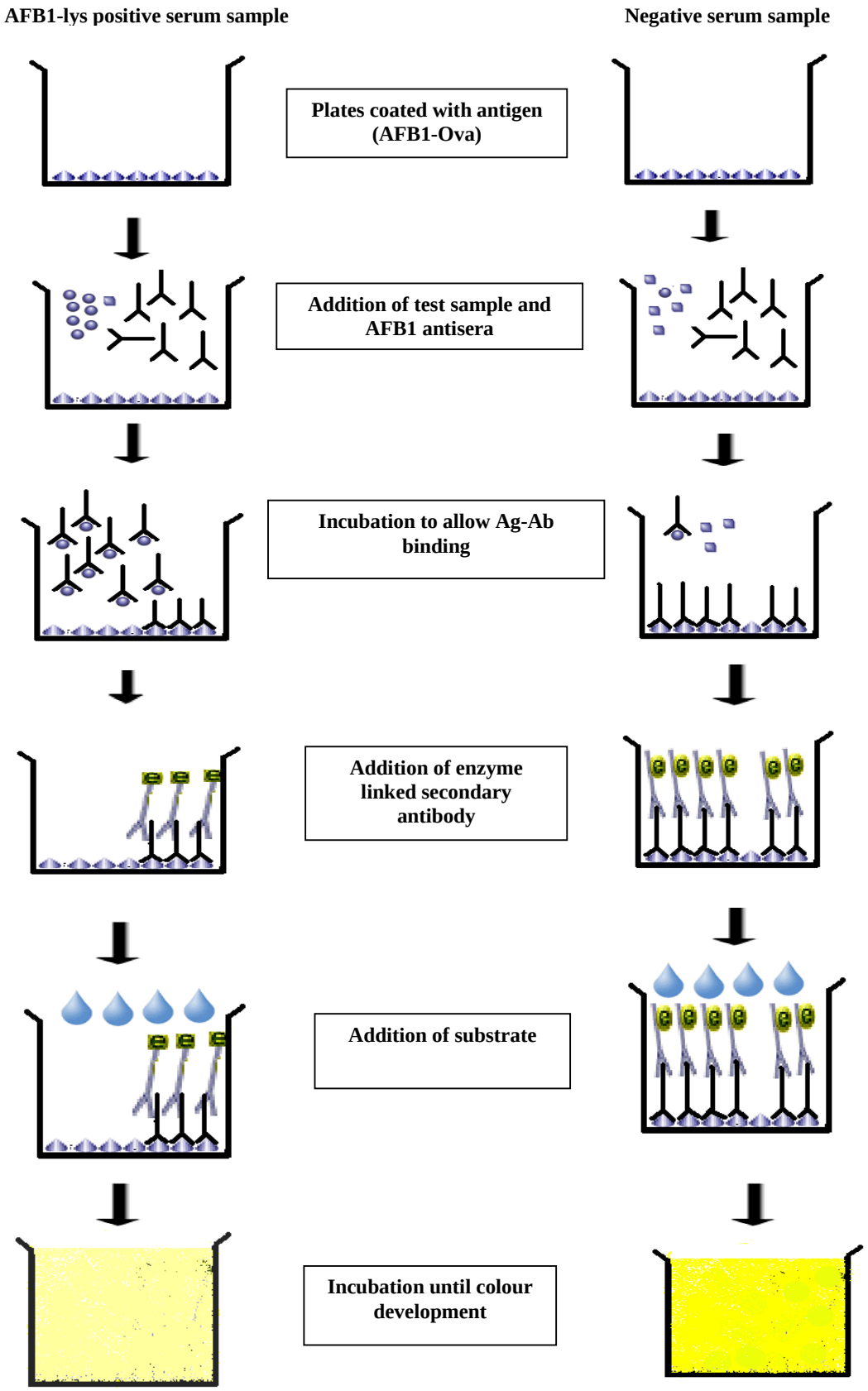
Previously, AFB1-lysine adduct was prepared by conjugating 8,9-dihydro-8,9-dibromo-AFB1 (AFB1-dibromo) with N- acetyl lysine for *in vitro* reaction instead of AFB1-epoxide (Sabbioni and Sepai, 1998). Preparation of later is simpler and more convenient and is widely used (Fig. 3).

Figure 3. **AFB1-lysine conjugate** (figure provided by Dr. Sabbioni through mail)



Molecular Weight =472.50 g/mol
Molecular Formula =C24H28N2O8

Figure 4. Indirect competitive ELISA for AFB1 positive and negative samples



2.9. Management of aflatoxins in plants and human

Human and animal exposure to aflatoxins can be reduced by mitigating aflatoxin contamination in food and feed (Turner *et al*., 2005). However, aflatoxin contamination is a complex problem and is influenced by diverse factors such as cropping practices, climate and socioeconomic background of the people (Waliyar *et al*., 2005b). Aflatoxin contamination of food is severe after long-term crop storage because of excessive heat, humidity; insect and rodent damage resulting in proliferation and spread of fungal spores. The main crops affected include maize and groundnuts, which, together, are the staple diet in many parts of sub-Saharan Africa. Groundnut is the most important oilseed crop in developing countries. Groundnut seed is the valuable source of protein for human and animal nutrition. India and China alone contribute to nearly 2/3rd of the world production.

The traditional approach to preventing exposure to aflatoxin has been to ensure that foods consumed have the lowest practical aflatoxin concentrations. In developed countries, this has been achieved for humans largely by regulations that have required low concentrations of the toxin in traded foods. However, this approach has certain limitations and clearly has failed as a control measure for developing countries. In developed countries, where regulations allow higher aflatoxin concentrations in animals, agricultural industries have developed alternative methods like chemoprotection and enterosorption to limit biologically effective exposure without the high cost of preventing contamination (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 feeding industry (Williams *et al.,* 2004).

It is well understood that much of the contamination of commodities with aflatoxin occurs during storage. To preserve aflatoxin production during storage, it is necessary to prevent growth of fungus through adequate drying (<10% moisture), elimination of insect activity that can increase moisture content through condensation of moisture resulting from respiration, low temperature, and inert atmospheres. Conditions needed

to prevent aflatoxin contamination are known, but is not always easy to produce them in storage systems in developing countries. Most people in rural areas grow and store their own food; in consequence, most food is stored in small, traditional granaries and there is little investment in the management of ideal storage conditions. Studies of grain quality in such storage structures show a steady increase in the aflatoxin content over time, which reflect the failure to maintain appropriate conditions (Turner *et al*., 2005).

Several approaches can prevent aflatoxin exposure in developing countries. Because much food contamination occurs during post harvest storage, methods to remove nuts or kernels damaged by fungus before storage and to restrict humidity during storage could reduce fungal growth and toxin production. The possible options for pre and post harvest prevention of aflatoxin contamination and the ICRISAT's management strategy of pre- and post-harvest contamination has given in Table 5 and Figure 5, respectively (Waliyar *et al.,* 2005b).

Turner *et al.* (2005) demonstrated a method for reduction in exposure to carcinogenic aflatoxins by post harvest intervention measures in West Africa. They selected a few villages, which have similar climate as well as same practice of groundnut cultivation, harvesting and storage. In 'control villages' farmers were left to follow their usual post harvest practices. In 'test villages', they were given a package to follow. This included hand sorting- damaged kernels which were removed and discarded before storage. Modification to usual drying procedures (drying on mats - spread on the ground for sun drying) make ground nut susceptible to humidity and difficult to gather in the event of unexpected rain. Therefore, locally produced natural-fiber mats were used for the sun drying process. Incomplete sun drying leaves residual humidity in the groundnuts during storage. Farmers were shown how to judge the completeness of sun drying by shaking the kernels to listen for the free movement of the dried nuts. Use of plastic or other synthetic bags for storage, rather than natural fiber bags promotes humidity. So, they provided the natural fiber jute bags. Storing the bags of groundnut bags on the floor leads to the risk of attracting humidity. Hence, to avoid that, wooded pallets were provided, on which the bags were kept. Insecticides were provided for sprinkling in small quantities on the floor of the storage facility under the wooden pallets at the start of the storage and intermediately afterwards. To

monitor the effectiveness of the intervention study, blood sample were taken and aflatoxin–albumin adduct level were measured by ELISA.

Table 5. A few options to reduce pre- and post-harvest contamination in crops
(Waliyar *et al*., 2005b)

Method	Purpose
I. Primary prevention	***To minimize fungal infestation and aflatoxin contamination***
▪ Cultivation of *A. flavus* resistant varieties	▪ Potential for control of fungal invasion and toxin production during crop growth.
▪ Control of field infection by following appropriate phytosanitary measures to reduce the fungal inoculum	▪ Limit fungal inoculum in the field
▪ Seed treatment and application of fungicides	▪ Limit fungal invasion during crop growth
▪ Appropriate scheduling for planting, harvest and post harvest	▪ Avoid drought stress and other abiotic stresses
▪ Application of soil amendments (gypsum, farmyard manure etc	▪ Enhancing soil nutrient (especially calcium) and water holding capacity, promoting the growth of antagonistic native soil-microflora
▪ Lowering moisture content of seeds after harvesting and during storage	▪ Limit fungal invasion and growth during storage
▪ Preservatives to prevent insect infestation and fungal contamination during storage	▪ Limit fungal invasion during storage
II. Secondary prevention	***Elimination or limiting the fungal contamination***
▪ Sorting of contaminated pods and kernels	▪ Reducing aflatoxin contamination in final product
▪ Re-drying the groundnut pods and kernels	▪ Limit further mold invasion during storage
▪ Appropriate storage conditions to avoid favorable conditions for mold growth	▪ Limit further mold invasion during storage
▪ Detoxification of contaminated product	▪ Chemical inactivation of aflatoxins through use of detoxification clay, ammonification, electronic sorting of kernels.

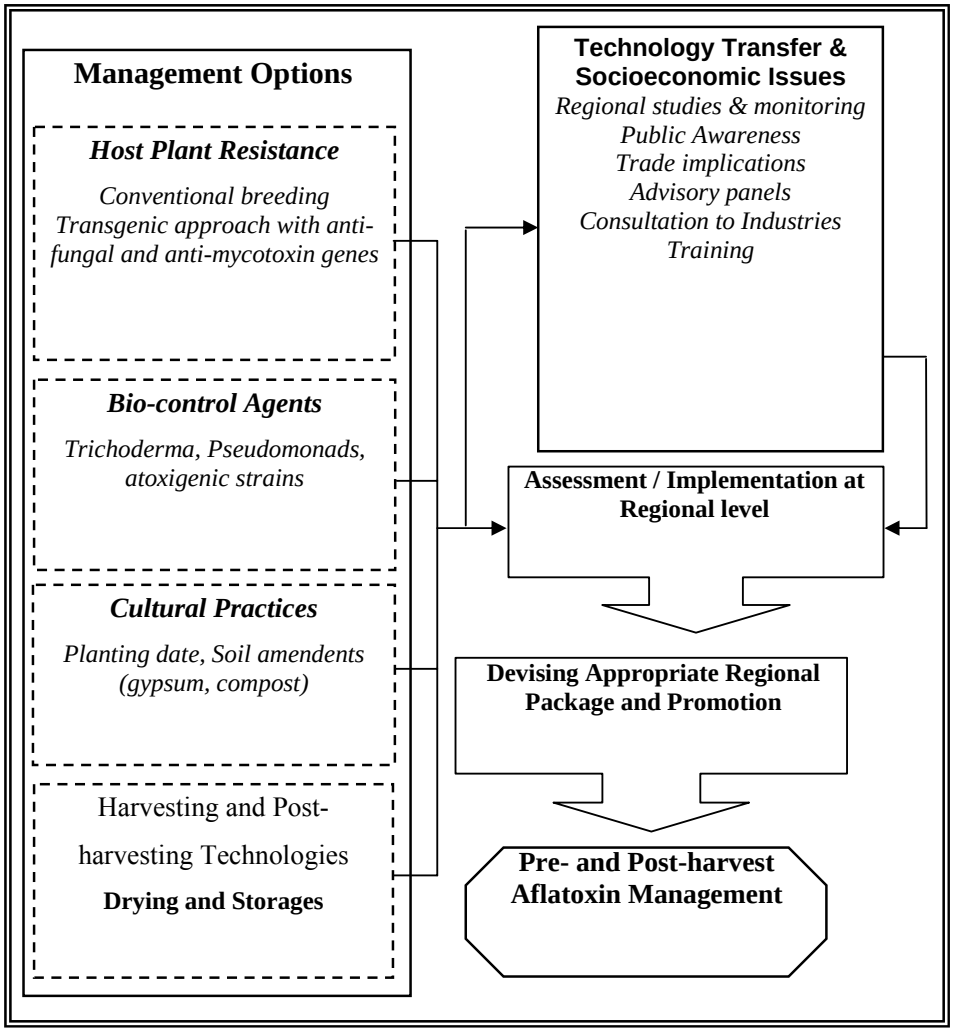
Since *Aspergillus* and aflatoxin contamination were already present in the field but then increase during storage, the post harvest adduct concentration was same in both 'test' and 'control' villages. But, during storage, the concentration increased in 'control' villages compared to the 'test' villages. In 'control' villages the mean adduct level increased during 5 months of storage from 5.5 pg mg^{-1} (immediately post harvest) to 18.7 pg mg^{-1}. In contrast, the mean adduct concentration in the 'test'villages after 5 month of storage was much the same as that immediately post-harvest (7.2 pg mg^{-1} to 7.0-9.0 pg mg^{-1}). At the end of the study period, mean concentration in the 'test' villages was less than 50% of the 'control' villages. The numbers of individuals exposed to toxin were also studied. After the 5-month storage period, only five (2%) people had non detectable concentration in 'control' villages compared with 47 (20%) of those in the 'test' villages.

The community-based intervention has shown to have a striking reduction in aflatoxin exposure by use of simple, low technology post harvest practices in a rural subsistence-farming community in Africa. Exposure was more than halved 5 months after harvest in individuals from the intervention villages. Moreover, only 2% of individuals in the control villages had undetectable concentrations of aflatoxin-albumin adducts compared with about 20% in the intervention villages (Turner *et al*., 2005). Such studies are necessary in developing countries in Asia to assess the risk of aflatoxin exposure and to implement suitable intervention strategies.

Diekman and Green, (1992) have given the following suggestions to prevent mycotoxin contamination of feed stuffs:

- Controlling the environmental factor that influence the growth of the fungus during storage i.e., moisture content of grain should be <14%
- Relative humidity should be <70%; Temperature should be –2.2 centigrade
- Oxygen availability should be <0.5% and also use of mold inhibitors and anti caking additive can reduce the fungus growth
- The technique like floating separation can remove the fusarium–infected kernels that are lighter than sound kernel
- Addition of 0.5% hydrated sodium calcium aluminosilicate in formulated feed can reduce the fungal infection

Figure 5. Schematic representation of integrated management strategy for pre and post-harvest management of aflatoxins in groundnut (Waliyar *et al*., 2005b)



ICRISAT's integrated approach for aflatoxin management for enhancing trade and human health is given in Fig 6. Anitha et al. (2007) initiated a 'Top-Down Strategy (TDS)' to prevent human exposure to aflatoxins (Fig.7). It involves identification of the most vulnerable groups who are at the risk of frequent exposure to aflatoxins. This is done by estimating the concentration of AFB1-lysine (a metabolite of aflatoxin in humans) in albumin fraction of blood, which has been shown to correlate with dietary aflatoxin intake over the previous 2-3 months (Turner *et al.,* 2005). Subsequently, sources of dietary contamination would be identified by analyzing the agriculture and food production practices in the region to formulate a risk prevention strategy.

Figure 6: ICRISAT's integrated approach for aflatoxin management for enhancing trade and human health. (Prepared by Dr.Lava Kumar in 2007)

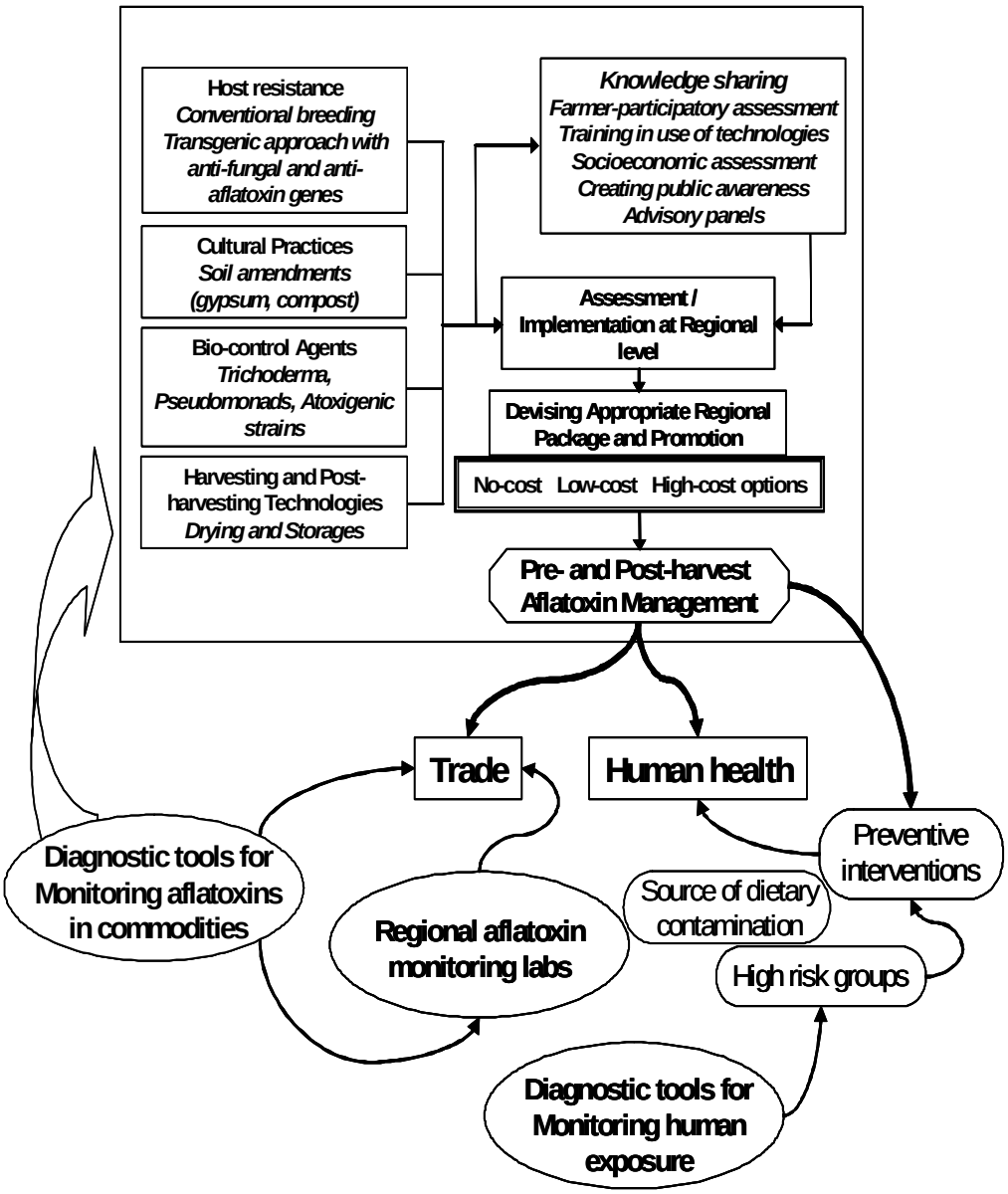
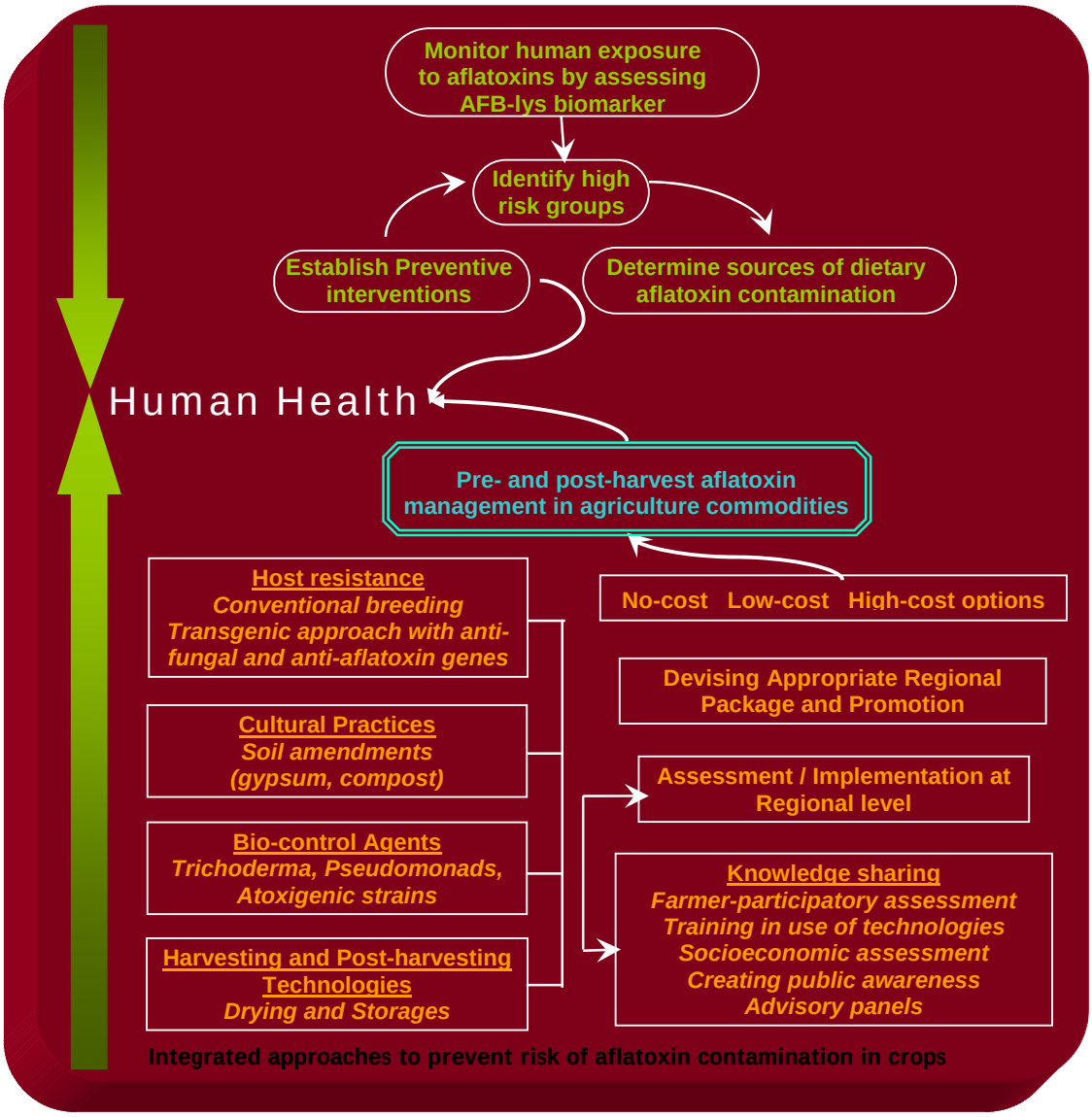


Figure 7. Schematic representation of Top-down strategy (TDS) and Bottom-up strategy (BUS) for preventing human exposure to aflatoxins (Anitha *et al.*, 2007)



3. MATERIALS AND METHODS

3.1. Production and characterization of polyclonal antibody

3.1.1. Equipment, reagents and antibodies

- Aflatoxin B1 (Sigma)
- Bovine serum albumin (BSA) (Sigma)
- Meta chloroperbenzoic acid (MCPBA) (ICN, Biochemicals, Ohio)
- Dichloromethane (DCM) (ICN, Biochemicals, Ohio)
- TLC plate- precoated, size (20 x 20cm), particle size 2-25 m Fisher Scientific's
- Anti-rabbit antibody conjugated with alkaline phosphatase (AR-ALP) (Sigma)
- General chemicals for buffer preparations were obtained from Sigma, BDH and Qualigens.
- ***Preparation of buffers for ELISA are given in the Appendix***

3.1.2. Preparation of AFB1- BSA adduct (Immunogen)** (Sujatha *et al.,* 2001)

1. In 500 µl of DCM, 11.36 mg of MCPBA was added and this was saturated with 0.1 M phosphate buffer. In another 500 µl of DCM 1550 µg of AFB1 was dissolved and mixed with MCPBA solution prepared in DCM. This was kept for gentle shaking at 5 ºC for 6 h for the formation of epoxide.
2. Ten mg of BSA was dissolved in 500 µl of 0.1 M phosphate buffer and this was added to AFB1 epoxide and incubated overnight at 5 ºC with gentle shaking.
3. The reaction mixture was centrifuged at 2,500 rpm for 5 min. The aqueous fraction was separated from the DCM fraction and washed thrice with DCM to remove traces of unreacted AFB1.

3.1.3. Estimation of AFB1-BSA adduct concentration

The AFB1-BSA adduct concentration was estimated using spectrophotometer (Beckman DU530). Phosphate buffer was used as a blank control. The OD at 595 nm was measured. Phosphate buffer was used as a blank. (Bradford, 1976)

Blank: To 1.8 ml of Bradford reagent 200 µl of PBS pH 7.4 was added. This was used for blank during spectrophotometric measurements.

Sample: Five µl of albumin was diluted to 200 µl in PBS and to this 1.8 ml of Bradford reagent was added and mixed well and readings were taken at 595 nm in a spectrophotometer.

3.1.4. Immunization of rabbit

AFB_1-BSA was used as an immunogen to produce antiserum. For immunization, 250 µg AFB_1-BSA in 250 µl of 0.1 M phosphate buffer, pH 7.2, was emulsified with an equal volume of Freund's complete adjuvant and used for immunization via intramuscular injections at multiple sites into a New Zealand White inbred rabbit. Four subsequent injections were given at weekly intervals using AFB_1-BSA emulsified in equal volumes of Freund's incomplete adjuvant. The rabbit was bled for polyclonal antiserum a week after last injection for four weeks at weekly intervals. To improve the antibody titer, a week after the 4^{th} bleed, the rabbit was given a booster immunization with AFB_1-BSA emulsified in incomplete Freund's adjuvant. After two weeks of rest, the animal was bleed for polyclonal antiserum at weekly intervals for eight weeks.

The titer of each bleed of antiserum was determined by IC-ELISA performed in 96-well microtiter plates (Maxi-sorp, Nunc) as described below.

3.1.5. Monitoring antibody titers

An indirect ELISA procedure similar to that reported for aflatoxin-albumin (Wild and chappot 1991) was used. Microtiter plates were coated with 10 ng mL^{-1} of aflatoxin-ovalbumin in carbonate buffer pH 9.6 and incubated at 37˚C for 2 hrs. Antiserum was diluted in PBS containing 0.05% Tween 20. Antiserum dilutions (from 10,000 to 1: 75000) in 50 µl were added to 100 µl of AFB1-lysine ranging from 100 ng to 100 pg.

goat antirabbit immunoglobulins conjugated to alkaline phosphatase were used at a 1:1000 dilution to detect antibodies attached to AFB1-lysine. p-nitrophenyl phosphate was used as a substrate at 1mg ml^{-1} and allowed to develop for 1 hr at room temperature. Absorbance was recorded at 405nm with an ELISA plate reader.

3.1.6. Characterization of antibody

3.1.7.1. DAC-ELISA to check cross-reaction of antisera

DAC-ELISA was performed to assess the antibody cross reaction. Plate was coated with 100 and 25 ng of AFB1-BSA, BSA and ovalbumin and it was incubated overnight at 4˚C. Plates were washed and AFB1 epoxide-lys and AFB1 antiserum at 1:2000 and 1:60,000 dilution, respectively, were added. ELISA plate was incubated at 37 ˚C for one hour. After washing, AR-ALP was added at 1:2,000 dilution and incubated for 1 h. Finally pNPP substrate was added at 1 mg ml^{-1} concentration; and the colour development was read at 405 nm.

3.2. Standardization of ELISA method to detect AFB1-lysine and Validation of ELISA method developed

3.2.1. Preparation of standards and coating antigen

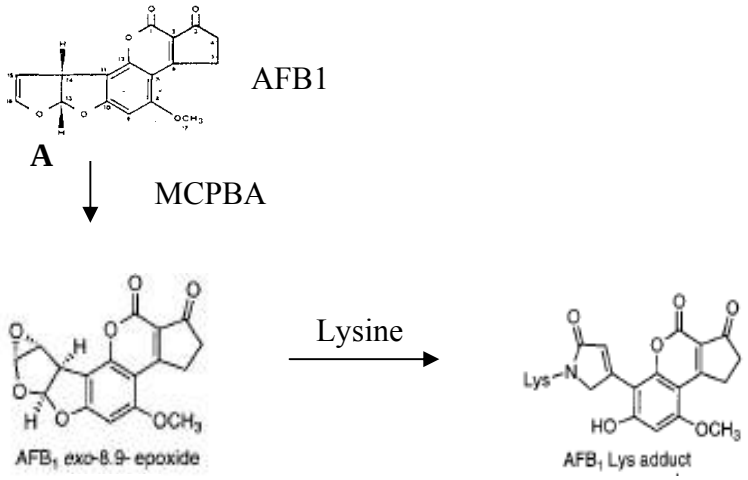
AFB1-Lysine adduct prepared invitro was used as a standard and AFB1-ovalbumin adduct prepared invitro was used as coating antigen. The preparation of coating antigen and AFB1-Lysine standard is given bellow:

3.2.2. Preparation of AFB1-lysine adduct

AFB1-lysine (Fig. 8C) adduct was prepared in subdued light. Protocol given in Sujatha *et al.* (2001) with some modifications was used for the preparation of adduct. First, AFB1 molecule was converted into epoxide (AFB1-8,9-epoxide; Fig. 8B) by oxidation with metachloroperbenzoic acid (MCPBA) (ICN, Biochemicals, Ohio) and it was used for conjugation with N-α-acetyl lysine (Sigma) to prepare AFB1-lys adduct as stated below.

1. Six mg of MCPBA was dissolved in 2.5 ml of dichloromethane (DCM) and the solution was saturated with 0.1 M phosphate buffer. To this 1 mg of AFB1 was added and incubated with gentle shaking at 5 ^{o}C overnight for the formation of AFB1-8,9 epoxide.
2. Ten mg of N-α- acetyl lysine was dissolved in 2.5 ml of 0.1 M phosphate buffer and this was added to the AFB1-8,9 epoxide and incubated overnight at 5 ºC with gentle shaking to allow the formation of AFB1-lys adduct .
3. This mixture was centrifuged at 2,600 rpm for 5 min (Labofuge 400e, Heraeus instruments) to separate aqueous fraction (which contains AFB1-lys) from the DCM fraction.
4. The aqueous fraction was washed thrice with DCM to remove residual AFB1.
5. Aqueous and DCM fractions were analyzed by TLC to verify adduct synthesis.

Figure 8. Structure of AFB1-8,9-epoxide and AFB1-lys (source Guengerich et al., 2002)



3.2.3. Preparation of AFB1- ovalbumin adduct (Sujatha *et al.*, 2001)

4. In 500 µl of DCM, 11.36 mg of MCPBA was added and this was saturated with 0.1 M phosphate buffer. In another 500 µl of DCM 1550 µg of AFB1 was dissolved and mixed with MCPBA solution prepared in DCM, and this was kept for gentle shaking at 5 ºC for 6 h for the formation of epoxide.
5. Ten mg of ovalbumin was dissolved in 500 µl of 0.1 M phosphate buffer and this was added to AFB1 epoxide and incubated overnight at 5 ºC with gentle shaking.
6. The reaction mixture was centrifuged at 2,500 rpm for 5 min. The aqueous fraction was separated from the DCM fraction and washed thrice with DCM to remove traces of unreacted AFB1.

3.2.4. TLC to estimate the adduct formation

Formation of AFB1-lys and AFB1-ovalbumin adducts were verified by TLC. For this each silica gel precoated TLC glass plate (20×20 cm) was activated for one hour at 110 ºC before use. The aqueous fraction, DCM fraction, N-α-acetyl lysine, ovalbumin and AFB1 standards were spotted on TLC. The plate was developed in a chloroform : acetone (9:1) solvent. The developed plate was air-dried and visualized under long-wave ultraviolet (UV) light.

3.2.5. Estimation of AFB1-lys and AFB1-ova adduct concentration

The AFB1-lys adduct concentration was estimated using spectrophotometer (Beckman DU530). Phosphate buffer was used as a blank control. For ELISA standards the concentration of AFB1-lysine is determined using the extinction coefficient of Sabbioni (1990). i.e at pH 7.4 400nm ε = 30 866, at 346 nm ε =15821 at 257 nm ε = 16927 and at 230 nm ε = 16200.

Similarly AFB1-ova adduct was also analyzed spectrophotometrically and its concentration was estimated by Bradford method and readings were taken at 595 nm. Phosphate buffer was used as a blank. (Bradford, 1976; Detailed in section 3.9)

3.2.6. Evaluation of AFB1-lys adduct and AFB1-ovalbumin adduct in ELISA

ELISA was performed typically as described in Waliyar *et al.* (2005a) with modifications as detailed here (ELISA buffer preparations given in Appendix). Antisera used for ELISA was produced in rabbits against AFB1-epoxide conjugated to BSA. Alkaline phosphatase conjugated anti-rabbit antibodies (AR-ALP) were used as secondary antibody and pNPP was used as substrate. The colorometric reaction was measured in an ELISA plate reader fitted with 405 nm filter.

To determine the working concentration of AFB1-ova adduct, 100 µl of AFB1-ova adduct was coated to the ELISA plate, with a starting concentration of 100 ng to 0.78 ng ml^{-1} and incubated overnight at 4˚C. Plates were washed and blocked with blocking buffer and incubated for 1 hr at 37 ^{o}C. Then 100 µl of the antisera was added at 1:40,000 dilution to each well and in subsequent step 100 µl of antirabbit antibody was added at 1:2,000 dilution, then 100 µl of pNPP substrate at 1 mg ml^{-1} concentration was added and the colorometric reaction was read at 405 nm.

To determine the working concentration of AFB1-lys, ELISA plates were coated with 100 µl of 10 ng AFB1-ovalbumin adduct and incubated at 4˚C for overnight. Plates were washed thrice with washing buffer and blocked using the blocking buffer for 1 h at 37˚C. Then, plates were washed thrice and 100 µl of AFB1-lysine standards (serial dilutions starting from 2000 pg to 7.8 pg ml^{-1}) prepared in PBS was added; and 50 µl of antisera against AFB1 epoxide (produced at ICRISAT) was added at 1:40,000 dilution and incubated for 1 hr at 37 ^{o}C. After washing the plates, 150 µl of antirabbit antibody (AR-ALP) was added at 1:2,000 dilution, then 150 µl of pNPP substrate at 1mg ml^{-1} concentration was added and the colorometric reaction was read in an ELISA plate reader fitted with 405 nm filter.

Data was entered into XL software (Microsoft Office 2000) and regression analysis was done using software Sigma Plot V 2 (SigmaPlot Inc.)

3.2.7. IC-ELISA protocol

Wells of the ELISA plates were coated with 150 µl of 10 ng/ml AFB_1-ova in 0.2 M carbonate coating buffer, pH 9.6, and incubated overnight at 4°C. The wells were replaced with 0.2% skimmed milk in PBS containing 0.05% Tween-20 (PBST) to block free sites of the well. In each subsequent step, plates were incubated at 37°C for 1 hr followed by three washes with PBST. Hundred µl of AFB_1-lys standards ranging from 7.8 to 2000 pg/ml prepared in PBS were added in duplicate wells, and in the same wells 50 µl of 1:40,000 antiserum (8th bleed) in PBST containing 1.5% BSA (PBST-BSA) were added. Sample wells consisted the same, except that, instead of the standard, 20 µl of hydrolyzed albumin (described below) in 80 µl of PBST was added. This was followed by addition of 150 µl of ALP-labeled anti-rabbit IgG at 1:2,000 in PBS. In the final step, 1 mg/ml para-nitrophenyl phosphate in 10% diethanolamine buffer, pH 9.8, was added and plates were incubated at 37ºC for 1 to 3 hrs. Optical densities (OD) were read in an ELISA plate reader (Multiskan plus, Labsystems) fitted with 405 nm filter. Recordings were taken till OD values of the control well reached 1.5 ± 2.0 OD. The flow chart for the ELISA for testing of AFB1 in serum has given in Fig 9 and Standard ELISA template for estimating the AFB1-lys in serum samples is given in Fig 10.

Figure 9. Flow chart of indirect competitive ELISA for the estimation of AFB-lys in serum samples.

ELISA plates were coated with 10 ng 1000 μl^{-1} of AFB1-ovalbumin conjugate diluted in coating buffer and incubated for two hours at 37°C.

↓

Washed the antigen-coated plate with washing buffer three times and the wells were blocked with skimmed milk for 1 hour at 37°C

↓

Washed and 100 µl of AFB1-lysine (7.8 to 2000 pg ml^{-1}) in PBS was added to the standard wells and 80 µl of PBS, 20 µl of sample against standard wells were added. Finally 50 µl of Antisera in PBS at 1:40, 000 dilution was added to all the wells and incubated for 1hr at 37°C with shaking.

↓

Wash and 150 µl of alkaline phosphatase labeled anti-rabbit IgG at 1:2000 dilution was added and incubated for 1hr at 37°C.

↓

150 µl of the substrate (p-nitrophenyl phosphate) in 10% diethanolamine was added to each well and the color development was read at 405nm in ELISA reader.

The concentration of AFB1-lysine were calculated with the following formula:

$$\frac{A \times D \times E}{G} \text{ pg mg}^{-1} \text{ albumin}$$

A= Concentration of AFB1-lysine in diluted or concentrated sample extract (pg ml^{-1})

D= Times dilution with buffer

C= Times concentration after clean up

E= Extraction solvent volume used (ml)

G= Sample weight (mg)

Figure 10. Standard ELISA template for estimating the AFB1-lys in serum samples

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B*	**Standard**	1	2	3	4	5	6	7	8	9	**Control**	
C	**Standard**	1	2	3	4	5	6	7	8	9	**Control**	
D	**Sample**	X1	Y1	Z1	B3	N1	M4	L1	O1	P4	U2	
E	**Sample**	X1	Y1	Z1	B3	N1	M4	L1	O1	P4	U2	
F	**Sample**	A1	A3	B1	B2	N2	M1	K1	O3	P3	H1	
G	**Sample**	A2	A3	B1	B2	N2	M1	K1	O3	P3	H1	
H												

Note: Edge wells (Rows A and H; and columns 1 and 12) were not used to avoid edge-row effect on the data. Control wells consist of only antiserum but not AFB1-lys.

Step 1: All wells of the plate were coated with AFB1-ova standard at 10 ng 100 μl^{-1}

Step 2: Plate was blocked with blocking buffer

Step 3.1: AFB1 epoxide- lys standard (2000 pg to 7.8 pg 75 μl^{-1}) and AFB1 epoxide antiserum (at 1:40,000) was added in the rows B and C (standards)

Step 3.2: Samples (hydrolyzed albumin from test serum samples) and antiserum was added in rows D and E; and F and G.

Step 4: AR-ALP (1:2000) was added into all the wells

Step 5: pNPP substrate (1 mg ml^{-1}) was added into all wells

Step 6: ELISA readings were taken at 405 nm

Step 7: Fed into computer. Absorbance values of standards were used for making a regression curve. From this, AFB1-lys concentrations in unknown samples were estimated.

3.2.8. High Performance Liquid Chromatography (HPLC) for validation of ELISA method (Sabbioni *et al.*, 1990)

HPLC were carried out using reversed-phase C18 column (Shimadzu Liquid Chromatography-LC-10AT VP) with a particle size 5 µm diameter linked to a fluorescence detector (Shimadzu RF-10 AXL). Solvent flow rate was 1 ml/min with mobile phase water:acetonitrile:methanol (70:17:17). Fluorescence detection parameters were set to excitation wavelength at 360 nm and emission wavelength at 440 nm. For each HPLC run, 20 µl of the hydrolyzed human serum albumin (HAS) was injected into the column and fractions were collected at 1 min interval. Each fraction was collected and dried in a vacuum evaporator and sample was reconstituted with 250 µl of PBS and analyzed in IC-ELISA.

3.3. Blood sample analysis for AFB1-lysine concentration

3.3.1. Equipment and reagents

- Microtitre plates – 96 well flat bottom plates, NUNC, Germany.
- Spectrophotometer- Beckman DU530, Beckman Coulter, USA.
- ELISA plate 96 well reader- Titrex Multiscan Plus
- TLC plate- pre-coated size (20 x 20cm), particle size 2-25 m (Fisher Scientific).
- Aflatoxin B1 standard (Sigma)
- Ovalbumin (Sigma)
- N-α-acetyl lysine (Sigma)
- Meta chloroperbenzoic acid (MCPBA) (ICN, Biochemicals, Ohio)
- Dichloromethane (DCM) (ICN, Biochemicals, Ohio)
- Diethanolamine (Sigma)
- P-Nitrophenyl Phosphate (pNPP) (Sigma)
- Anti-rabbit antibody conjugated with alkaline phosphatase (AR-ALP) (Sigma)
- General chemicals for buffer preparations were obtained from Sigma, BDH and Qualigens.
- Rabbit polyclonal antiserum to AFB1 epoxide produced at ICRISAT (Anitha *et al.,* 2011)
- **Preparation of buffers for ELISA are given in the Appendix (8.1)**

3.3.2. Sample selection:

Control and case (patient) samples were selected from hospitals based on inclusion and exclusion criteria

3.3.2.1. Criteria of inclusion:

Controls: Subjects from the same family who do not have liver disease and consuming same lot of food that the patient consumes. Control samples were also collected from general population who do not have family history of liver disease or a history of any genetic disease that may lead to liver disease.

Cases: Patients who were diagnosed with liver disease (fatty liver, cirrhosis of liver, Hepatitis B, Hepatitis C, Chronic alcoholic and non alcoholic liver disease and hepatocellular carcinoma).

3.3.2.2. Criteria of exclusion

Controls: Subjects with the history of liver diseases.
Cases: Patients who were not confirmed with the any liver disease.
Insufficient blood sample or missing of the data of the subjects.

3.3.3. Collection of blood sample

Samples were collected from patients having various liver diseases including fatty liver, cirrhosis of liver, Hepatitis B, Hepatitis C, Chronic alcoholic and non alcoholic liver disease and hepatocellular carcinoma from several hospitals in Hyderabad, Andhra Pradesh. The control samples were collected from the individuals of the same family with no history of liver disease.

Data of clinical and diet profile of each patient was documented using a form custom designed for the purpose (Annexure-I). All the objectives of the study were clearly explained to the subjects. A signed consent on an Ethics Committee Approved form was obtained from patients willing to participate. The demographic data, including (i) age (ii) family history of liver diseases (iii) habits like smoking, alcohol consumption, (iv) dietary profile and (v) clinical profile including liver function test, was obtained.

After collecting the blood sample serum was separated from blood by placing the tubes in slant position for 60 min and centrifuged at 2,500 rpm for 10 min. Serum was collected into a separate tube and heat treated at 60 ^{o}C to inactivate viruses and any infectious agents. These were then stored at –20ºC until further use. After use, samples were autoclaved and discarded.

3.3.4. Isolation of albumin from serum (Modified from Chapot and Wild, 1991; Vinitketkumnuen *et al*., 1997).

1. To the 500 µl of serum 750 µl of saturated ammonium sulphate was added drop wise. The mixture was vortexed and centrifuged in a Sorvall ss35 rotor at 9,000 rpm at 4 °C for 15 minutes to precipitate immunoglobulins.
2. Supernatant was collected and 100 µl of 1 M acetic acid was added to adjust its pH to 5, at this pH albumin precipitates from the serum. This was centrifuged at 9,000 rpm at 4°C for 15 minutes to collect albumin. The supernatant was discarded and the precipitated albumin was redissolved in 1 ml of phosphate buffered saline (PBS, pH 7.4) and stored at –20 °C till its use.

3.3.5. Estimation of protein concentration

The concentration of albumin was estimated by the Bradford method (Bradford, 1976).

Blank: To 1.8 ml of Bradford reagent 200 µl of PBS pH 7.4 was added. This was used for blank during spectrophotometric measurements.

Sample: Five µl of albumin was diluted to 200 µl in PBS and to this 1.8 ml of Bradford reagent was added and mixed well. Readings were taken at 595 nm in a spectrophotometer.

3.3.6. Albumin hydrolysis with Proteinase K (Modified from Chapot and Wild, 1991):

Albumin was hydrolyzed into amino acids by digesting with proteiases.

- Two mg of the albumin was digested with 0.67 mg of proteinase K in a total volume of 0.8 ml of PBS, pH 7.4 and incubated at 37 °C for 17-18 h.
- To this, 10 mg BSA (from 100 mg ml^{-1} stock) was added to precipitate the undigested sample and proteinase K.
- Proteins were subsequently precipitated by the addition of two volumes of cold ethanol. The tubes were gently shaken for few minutes and incubated at -20°C for 2 h.
- The precipitated proteins were removed by centrifugation at 5,000 rpm for 15 min. Ethanol was evaporated in a vacuum evaporator (Speed Vac Plus SC110, SAVANT, Refrigerated Vapor Trap RVT 400, SAVANT). The final volume was

made up to 0.8 ml with PBS and stored at –20°C until use for the estimation of AFB1-lys adducts by ELISA.

- Hydrolysis of albumin was confirmed by analyzing a fraction of digested sample by denaturing polyacrylaminde gel electrophoresis (12% SDS-PAGE, detailed in section 3.3.7).

3.3.7. SDS-PAGE for digested albumin

Undigested and digested albumin samples were analyzed by 12% SDS-PAGE (BROVIGA, mini slab gel electrophoresis apparatus). Procedure given by Kumar *et al*. (2004) was followed to prepare 12% resolving gel and 4% stacking gel (composition given in Appendix). Albumin and hydrolyzed samples were mixed with equal volumes of Lamelli buffer (Appendix) and kept in boiling water bath for 3 minutes and loaded into the wells of PAGE gel. After electrophoresis, gels were silver stained following the procedure given by Kumar *et al*. (2004) (detailed in Appendix 8.3).

3.3.8. Artificial spiking

In order to assess the sensitivity of the albumin extraction and hydrolysis procedure, the albumin (2 mg) purified from healthy serum was artificially spiked with different concentrations of AFB1-lys. The sample was digested with proteinase K, as per the methods given in the section 3.3.6 and hydrolyzed sample was assayed in ELISA to estimate AFB1-lys concentration.

3.3.9. ELISA for the AFB1-lysine spiked and digested albumin

Artificially spiked samples and controls (hydrolyzed albumin from normal serum sample) were analyzed in ELISA as described in section 3.3.10.

3.3.10. Testing of serum samples for AFB1-lys concentration using IC-ELISA

The blood samples obtained from the various Hospitals in Hyderabad were used for analysis. AFB1-lys and albumin were isolated from these samples and stored at –20ºC. Two mg of the protein from these samples were digested with 0.67 mg of proteinase K

and processed as mentioned in section 3.3.6. These samples were stored at –20ºC. ELISA was performed as mentioned in section 3.2.7 with 20 µl of test sample per well.

3.4. Production of monoclonal antibody

3.4.1. Equipment and reagents

- Iscove's Modified Dulbeccos Medium (IMDM) (Gibco BRL)
- Fetal Bovine Serum (FBS) (Gibco BRL)
- L-Glutamine (Glutamax-II, 200mM 100x) (Gibco BRL)
- Mercaptoethanol (50 mM 1000x)
- Penicillin (Sigma)
- Streptomycin (Sigma)
- Gentamycin (Sigma)
- Nystatin (Sigma)
- PEG 4000 (Merk)
- HAT supplement (50X, Gibco BRL)
- HT supplement (100X, Gibco BRL)
- Membrane filters
- CO2 incubator
- Immunized BALB/c mouse,
- Chloroform/ ether in a beaker with cotton
- ethanol
- Sterile blunt forceps and scissors (2 sets)
- 100 mm petridish
- Dissection pad with blotting paper and needles
- Syringe 1 ml 5 ml and 10 ml glass
- Needles 22G
- Sterile centrifuge tubes 2x50ml
- Eppendorf tubes
- Adult healthy mouse
- Glass syringe 10 ml
- Dissection instruments (blunt forceps, scissors, dissection pad and needles)

- 96 well culture plates
- ELISA plate (Nunc Maxisorb)
- Aflatoxin B1
- Keyhole Limpets Hemocyanin

3.4.2. Immunization

The immunogen Aflatoxin B1-Keyhole Limpets Hemocyanin (AFB1-KLH) conjugate (conjugate prepared using protocol given in section 3.1.2 by replacing BSA) was used to produce AFB1-KLH antibody. The immunogen was prepared and 250 µg of immunogen was emulsified with an equal volume of complete Freund's adjuvant (Sigma). The primary injection was given subcutaneously to 6 of the six weeks old female Balb/c mice. The subsequent injection was given with the interval of 21 days with the same antigen prepared in Freund's incomplete adjuvant. The mice were given rest for one month. Four days before fusion, an intraperitoneal booster dose was given with AFB1-KLH in PBS.

The immunogen was injected at 21-day intervals. Only two injections were given to the mice. Mice were screened by an ELISA for specific serum antibody induction, and the mice with the best specificity were given an intraperitonial boost (2.5 mg) of conjugate, 4 days before their spleens were removed.

3.4.3. Preparation of media

Iscove's Modified Dulbeccos Medium (IMDM) was used. It contains L-glutamine and 25mM HEPES buffer. As per the manufacture instruction. 3.024 gm. of NaHco3/lit of medium was added during preparation.

To 900 ml of sterile water one sachet of powder IMDM media and antibiotics - penicillin (100 U/ml), gentamycin (50 µg/ml), and streptomycin (100 µg/ml) were added, stirred thoroughly and sterilized immediately by membrane filtration. Nystatin (5U/ml) was added after sterilization. As L-Glutamine is unstable, 2mM of glutamine (from 200 mM stock) was added every 15 days to the medium. Prepared medium was stored in the

dark to prevent the production of highly toxic photoproducts. 10% and 20% IMBM were prepared for myeloma cell growth and hybrid cell growth using FBS.

3.4.4. Preparation of myeloma cells:

The myeloma cell line, Sp2/O was grown at 37C in 5% CO2 incubator. Cells were usually maintained in T25 flasks in 5 ml of 10% IMDM. When the cells were confluent, they were diluted in fresh medium and distributed in additional flasks.Three days before fusion, the cells were split every day. One day before fusion, the cells were split into the fresh medium supplemented with 10% IMDM. The growth of the cell was ensured to be in log phase before being used for fusion.

3.4.5. Preparation of splenocytes

Mice were sacrificed using chloroform. Then, they were placed on their backs for dissection. The mouse was swabbed with 70% ethanol before incision. Immediately after cutting, the heart blood was collected quickly by making a single puncture. Then, the spleen was removed aseptically from the immunized mouse and placed in a 100 mm tissue culture plate. Using a 10 ml glass syringe fitted with 22G needle, the serum free IMDM media was injected into the spleen, such that splenocytes are seen coming out and become dispersed. This process was repeated several times to obtain the maximum number of the cells in the medium. The collection of cells was stopped once the spleen become pale in color. The medium containing dispersed cells was transferred to a sterile 15 ml centrifuge tube leaving behind the tissue pieces. The cells were centrifuged at 1,500 rpm (400g) for 10 minutes. The supernatant was discarded and the pelleted cells were washed by re-suspending it in 10 ml serum free IMDM medium.

3.4.6. Counting the cells:

After second centrifugation, pelleted cells were resuspended in 10% IMDM. Then, 1:100 dilutions of splenocytes were made with lysis buffer (Appendix) and left at 37˚C for 5 minutes. After RBC lysis, the splenocytes were counted using a hemocytometer containing 16 square.

3.4.7. Preparation of macrophages (Feeder cells)

The mouse was sacrificed using chloroform. First, the animal was washed with 70% ethanol. Then it was placed on its back for dissection. Using a blunt forceps and scissors, a small cut was made in the lower abdominal region and skin was removed to expose the peritoneum. Serum-free cold IMDM medium was injected as much as possible, from a single site. Then, without removing syringe, the abdomen of the mouse was taped in such a way that the tissue macrophages entered into IMDM medium. Then, the needle was withdrawn with the liquid completely, without any tissue. The liquid was transferred to a 15 ml tube and centrifuged at 1,500 rpm (400g) for 10 minutes. The pellets were re-suspended in 20% IMDM medium and counted using a hemocytometer.

3.4.8. Fusion

The hybridoma cells were derived from fusion between myeloma cells (Sp2/O cell line) and splenocytes of Balb/c mice immunized with AFB1-KLH. The technique for the production of hybridoma cells was similar to Galfre and Milstein 1981. The splenocytes and myeloma cells were mixed in a single glass tube in a ratio of 5:1. For $10x10^6$ cells of spleenocytes $2x10^6$ cells Sp2/O cell were used. This cell count was used for one culture plate. The number of culture plates used for fusion experiment varies according to the cell population. The excessive splenocytes were stored in a freeze mix (Appendix) at -80˚C. The splenocyte and myeloma cells mixed in a right proportion (5:1) were centrifuged at 1500 rev/min for 10 minutes. The supernatant was discarded and 0.5ml of pre-warmed PEG was added slowly over a period of 1 minute (0.1 ml for every 6 seconds) by tapping the tube between each drop. In the following minute, the tube was tapped continuously allowing the cells to suspend completely in PEG. Then, 4.5 ml of serum free IMDM was added in 4 minutes to dilute the PEG. This was kept at 37˚C for 20 minutes to 1hr. Then, the cells were centrifuged at 1500 rev/min for 10 minutes. The supernatants were discarded. The fused cells were then suspended in a complete medium containing 20% IMDM, 100X HAT, macrophages, 1000X mercaptoethanol. This was distributed in 96 well culture plates in such a way that each well received 200 µl of medium containing cells. As control, myeloma cells were added in 1 well in HAT

medium. They should all be dead in 4 days. The fusion experiment is briefly described in the Fig 11.

3.4.9. Screening cells for antibody production

After 12 day culture, supernatants from each well of the culture plate were analyzed using DAC ELISA. Culture supernatant from the cells that gave absorption of 3 OD was transferred to 24 well culture plates in IMDM containing 20% FBS and 100X HT. The supernatants from 24 well culture plate were again tested by DAC ELISA. The clones which maintained absorption values over 1 were chosen for further selection. Cell suspensions from each well of the 24 well cultured plate were diluted to give approximately one cell per well when distributed into a 96 well culture plate. The plates were examined for the presence of number of hybridomas. Those that contained a single hybridoma in each well were retained and were screened for neutralization titers.

3.4.10. DAC ELISA

The ELISA plates were coated with 100 µl of AFB1-KLH conjugate per well in the concentration of 1µg/100 µl. After 1 hr incubation, 100 µl of culture supernatant were transferred to the ELISA plate. This was incubated for 1hr at room temperature and after washing, 100 µl of anti-mouse ALP conjugate was added in the concentration of 1:1000. This was incubated for 1 hr and finally 100 µl of 1mg/ml of PNPP substrate was added. Color development was observed and the read for optical density using ELISA plate reader at 405nm.

3.4.11. Multiplication of selected hybridomas

The cells were fed whenever the growth medium turned yellow by removing the spent medium with a pipette and replacing it with a fresh 20% IMDM medium. Spent medium containing monoclonal antibody was collected pooled and stored at -20˚C or at 4 ˚C with 0.02% sodium azide.

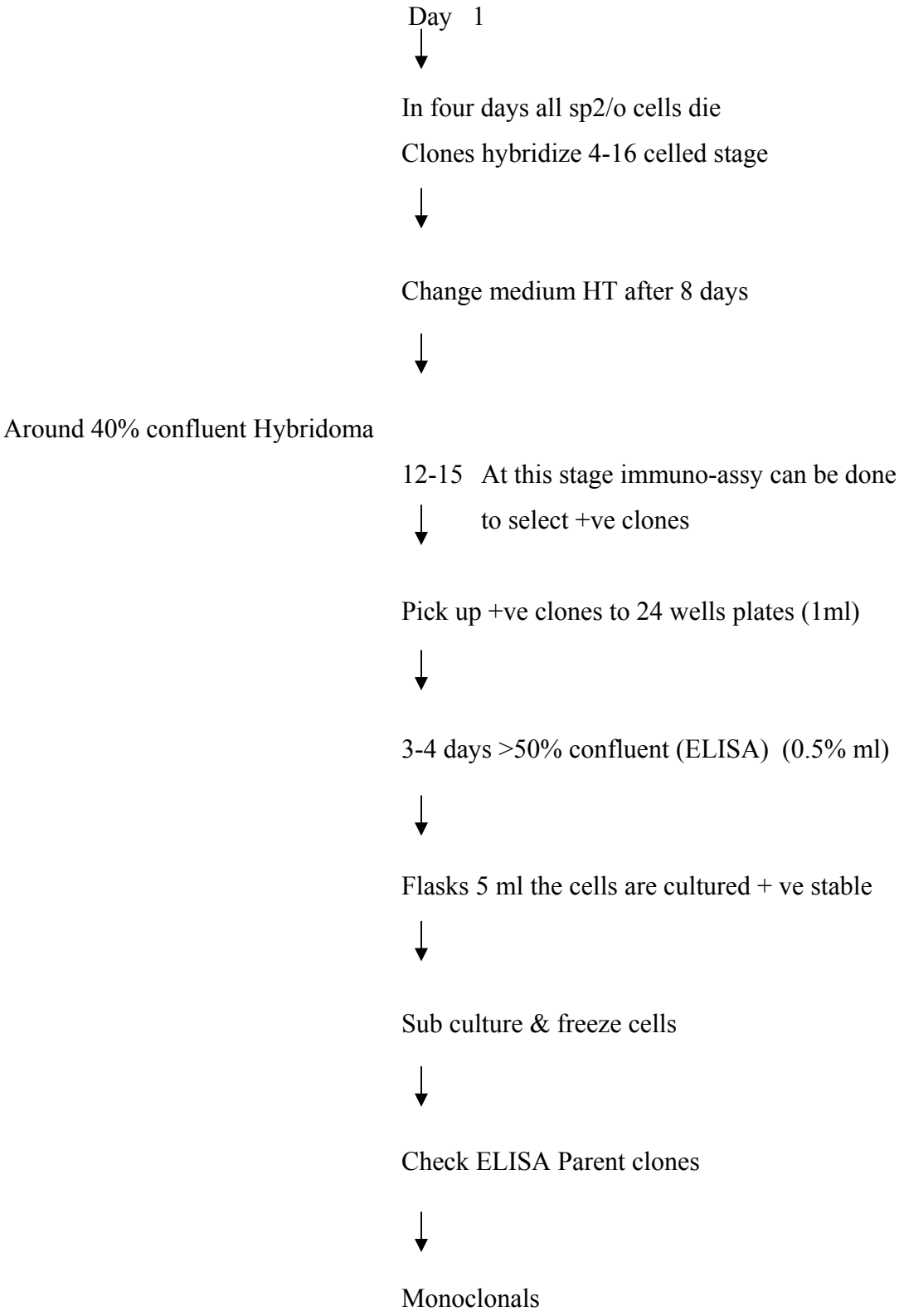
3.4.12. Cloning of hybridomas by limiting dilution

Cell lines that were positive in the DAC ELISA screenings were transferred to individual 5 ml culture flasks. These cells were grown in CO_2 incubator and screened again by ELISA for its antibody activity. The antibody in a supernatant was collected in a tube and stored as mentioned above the cells were scraped and centrifuged at 2000 rev/min for 5 minutes. The cell suspension in freezing medium was dispensed at 0.5ml per freezing vial. These vials are stored at -80˚C.

3.4.13. Determination of specificity of monoclonal antibodies (MAbs)

To evaluate the cross reactivity of each of the monoclonal antibodies, it was essential to determine the optimum condition for neutralization. These included coating antigen (AFB1-BSA) concentrations and the dilution of the antibody required for neutralization. Antibody titers were determined by the indirect competitive ELISA procedure described in the section 3.2.7. The optimum concentration required in each step to obtain the maximum sensitivity was determined by 50% displacement values of B/B0, where B is the extinction of the well containing AFB1 and B0 is the extinction of the well without toxin, derived from the slope of calibration curves. Using these parameters, dilutions of (50µl/well) of monoclonal antibodies were added to 100 µl of AFB1-BSA, at dilution ranging from 1:500 to 1:20,000. The protocol used for the characterization of antibody specificity was similar to those employed for the characterization of polyclonal antibodies. 3.1.7.1 and 3.2.7.

Figure 11. Fusion experiment



3.5 Detection of *p53* Mutation using Restriction Fragment Length Polymorphism (RFLP)

3.5.1. Equipment and reagents

- DNA extraction kit (Nucleospin)
- Horizontal electrophoresis apparatus (Geni)
- Thermocycler (Applied Biosystems)
- UV illuminator
- PCR reagents (Biorad)
- Primers (Sigma)
- All the chemicals for electrophoresis were procured from Sigma

3.5.2. Sample collection

One hundred thirty blood samples were collected from Hepatitis B surface antigen positive patients. These samples were obtained from Global hospitals and Apollo hospitals, Hyderabad, India. In addition, 108 samples were also collected from healthy people to use them as controls in the analysis. Sample selection, criteria of inclusion and criteria of exclusion is given in sections 3.3.2, 3.3.2.1 and 3.3.2.2 respectively. All of the patients were permanent residents of Andhra Pradesh and adjoining states of south India. For the extraction of DNA, 0.5 ml of blood samples was collected in EDTA vial. Samples were immediately transferred to ICRISAT's "Mycotoxicology and virology lab, Hyderabad under refrigerated condition. Then samples were stored at -70ºC for further use.

3.5.3. Extraction of DNA from blood sample

DNA was extracted from 200 µl of blood using Nucleospin kit as per manufactures protocol and stored at 4ºC until polymerase chain reaction (PCR) performs. The protocol for the extraction of DNA is as follows.

1. Blood samples were lysed by the addition of 25 µl of proteinase K and 200 µl of blood into 1.5 ml microfuge tubes

2. 200 µl of lysis buffer B3 was added to the samples and vortexed the mixture vigorously for 10 seconds
3. Samples were incubated at 70ºC for 10-15 minutes
4. 210 µl of ethanol was added to each sample and vortexed.
5. Nucleospin Blood column was placed in a 2ml centrifuge tube and loaded the sample and centrifuged for 1 min at 11,000g.
6. The flow through was discarded with collecting tube.
7. Blood column was then placed in to a new 2ml collecting tube and 500 µl of buffer BW was added and centrifuged for 1 min at 11,000g then collecting tube was discarded with flow-through.
8. Blood column was again placed in to a new collecting tube and 600 µl of buffer B5 was added and centrifuged for 1 min at 11,000g then discarded the flow-through.
9. Column was again kept in a same collecting tube and centrifuged for 1 min at 11,000g to dry the column.
10. The column was then placed on 1.5 ml microfuge tube and 100 µl of pre-warmed elution buffer BE (70ºC) was dispensed directly on to the silica membrane and incubated for 1min at room temperature then centrifuged for 1min at 11,000g.
11. DNA in elution buffer was collected and stored in -20 until use.

3.5.4. Agarose gel electrophoresis

The genomic DNA was analysed by 2% agarose gel electrophoresis using horizontal gel electrophoresis system. A gel slab of required size containing 2% agarose and 6µl of 10mg/ml ethidium bromide was prepared in 1x TBE using gel mould. After setting time of 20 min the gel mould was placed in a tank containing sufficient quantity of 0.5x TBE buffer (Appendix 8.5). The DNA along with DNA Marker (λ DNA Marker; Roche, Cat# 528 552) was loaded in wells of 2% agarose gel and electrophoresed at 100V for 1-2h. Removed the gel from the tray and visualized on a UV-transilluminator and photographed with a Polaroid^{R} camera fitted with a UV filter.

3.5.5. Optimization of PCR condition

After DNA extraction from blood samples PCR was carried out in Applied Biosystems thermocycler. The thermal cycles of the reaction were standardized by performing the PCR at various combinations of denaturation, annealing, and extension temperatures according to the Tm of the degenerate primers. We focused on the analysis of codon 249 by amplifying a region of exon 7 surrounding codon 249 using forward (*p53*Fser: 5'CTT GCC ACA GGT CTC CCC AA 3') and reverse (*p53*Rser: 5'AGG GGT CAG RGG CAA GCA GA 3') primers. The temperature profile followed for amplification of *p53* exons was initial denaturation at 94°C for 2 minutes followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, and a final extended synthesis at 72°C was performed for 5 minutes. DNA Marker (λ DNA Marker; Roche, Cat# 528 552) was used to determine the size of PCR amplified product. Gel was stained with ethidium bromide (0.5µg/ml), viewed on a UV-transilluminator and photographed with a polaroid^{R} camera fitted with a UV filter.

The PCR reaction mixture was standardized as follows: each 50 µl reaction mixture consists the following components:

Components	**Single reaction with total volume of 50 µl**
10x PCR reaction buffer	5 µl
$MgCl_2$ (25mM)	3 µl
Forward primer (10pm/µl)	2 µl
Reverse primer (10pm/µl)	2 µl
Taq polymerase (2.5U)	0.5 µl
dNTPs mixture (100mM)	1 µl
Template DNA (50-60ng/µl)	2 µl
Double sterile distilled water	34.5 µl
Total volume	**50 µl**

3.5.6. Purification of PCR products

To obtain enough amounts of DNA fragments for further testing, 1 µl of PCR product of each sample was picked up as template to have another 4 PCR reactions with the same

amplifying conditions. The PCR products were then purified as follows:

- To each 250 µl of PCR product, equal volume of phenol:chloroform was added and then centrifuged at 13,000 rpm.
- Supernatant was taken into another tube and equal volume of ethanol was added and kept for precipitation at -70°C for 1 hr.
- Then centrifuged at 13,000 rpm and pellet was kept for drying at 37°C for 10 min.
- 25µl of milli Q water was added to dissolve the pellet and stored at -20ºC for further applications.

3.5.7. Restriction Fragment Length Polymorphism (RFLP)

The 254 bp of purified DNA fragment, derived from exon 7 of *p53* gene, was subjected to restriction enzyme *Hae III* (New England Biolabs Company) digestion. The restriction enzyme digestion reaction system consists of 1.3 µl *HaeIII*, 2 µl 10×buffer, 6 µl PCR product, 0.7 µl ddH2O. These reaction mixtures were kept in the incubator at 37ºC for 1 hour. Enzyme *Hae III* cleaves a GG/CC sequence at codons 249-250, generating 92bp, 66bp and several small fragments from the 254 bp purified DNA product of the PCR reaction. If there is a mutation at codon 249-250 resulting in an uncleaved, 158bp fragment, and this feature will be distinguished from that of normal samples on 2 % agarose gel stained with ethidium bromide. Absence of the band at 254 bp (full-length PCR products) provides a control for complete digestion of the PCR product.

3.5.8. Statistical analysis

Pearson's chi-square and Fisher's exact test were used to assess statistical significance of frequency tables of independent variables and 249 Ser mutations with 95 % confidence intervals (CI) using the software SAS 9.2V to estimate the risk of mutation among the study groups considering age, gender, hepatitis B surface antigen status and aflatoxin B1 positive status in ELISA as potential confounders.

4. RESULTS

4.1 Production and characterization of polyclonal antibody

4.1.1. Immunization of rabbit

The immunization protocol followed in this experiment was good enough to elicit the immune response. The antibody obtained from rabbit serum was further characterized as indicated in the following sections.

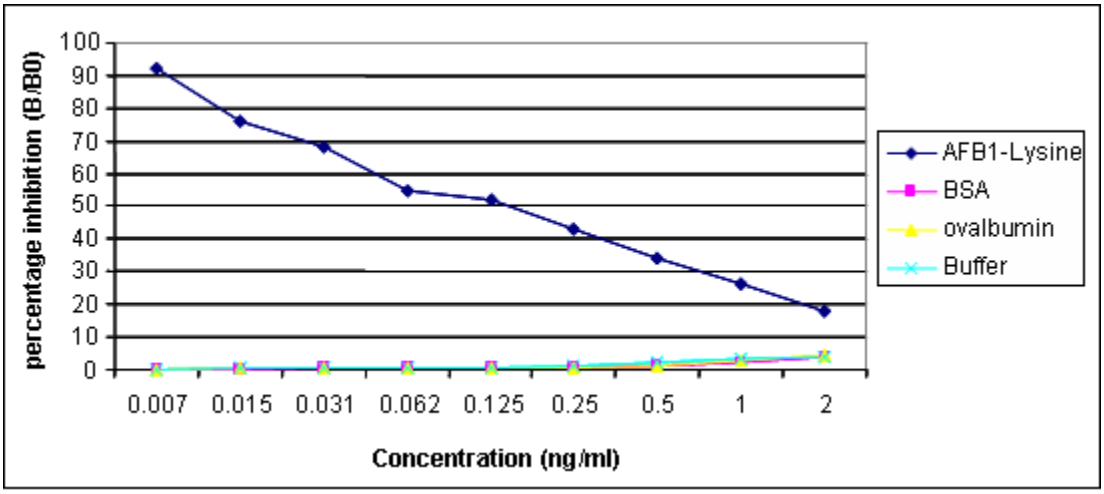
4.1.2. Monitoring antibody titer

The titer of each bleed of antiserum was determined by IC-ELISA performed in 96-well microtiter plates (Maxi-sorp, Nunc). The procedures used in this study have contributed to the production of high titered and specific antiserum against AFB1-lys, and high yields of synthetic AFB_1 adduct necessary as standards. Titer of the antiserum prior to booster immunization was between 1:2,000 to 1:10,000 and titer of antiserum post-booster injection was 1:40,000 to 1:75,000, suggesting positive effect of longer resting time and booster dose on improving the antiserum titer. Antiserum, used after cross absorption with 1.5% BSA, did not cross-react with carrier proteins, 10 mg/ml BSA or ovalbumin in PBS. Eighth batch antibody that had a titer of 1:40,000 was used in subsequent experiments. The optimum antiserum dilution required to obtain the maximum sensitivity was determined by 50% displacement values of B/B0, where B is the OD of the well containing AFB_1-lys, and B0 is the OD of the negative control, using various dilutions of the antibody was added to 100 µl of the AFB_1-lys dilutions ranged from 2000 pg/ml to 7.8 pg/ml. This demonstrated that antiserum diluted at 1:40,000 can detect up to 5 pg AFB_1/mg HSA.

4.1.3. ELISA for detecting cross reaction

Specificity of the antiserum determined by DAC-ELISA mentioned in 3.1.7.1 and IC-ELISA mentioned in 3.2.7, except by replacing AFB_1-lys with BSA and ovalbumin demonstrated lack of antibody cross reaction with carrier proteins (Fig. 12).

Figure 12. Evaluation of polyclonal antibodies (1:40,000 v/v) against AFB1-lys, BSA and ovalbumin in IC-ELISA.



4.2 Standardization of ELISA method to detect AFB1-lysine and validation of ELISA method developed

4.2.1. Preparation of AFB1-lys and AFB1-ova adducts

AFB1-lys adducts showed two peaks, one at 275 nm and other at 340 nm which was detected by spectrophotometer reading from 200-400 nm (Fig. 13). The concentration of the adduct was 55.5 μg ml^{-1}, which was calculated as mentioned in section 3.2.5. The concentration of AFB1-ovalbumin determined by spectrophotometer reading at 595 nm using Bradford method was 4.8 mg ml^{-1} (Fig. 14). The AFB1-ovalbumin and AFB1-lys adduct was aliquoted into 0.2 ml tubes and stored at –20 ^{o}C.

TLC analysis confirms the presence of AFB1-lys adduct and AFB1-ova adduct which showed a single fluorescence spot at the base of the plate with an R_f value equal to zero and there was no free AFB1 present in the aqueous phase (Figs. 15 and 16). Standard AFB1 showed single fluorescence spot at the top. The organic phase showed multiple fluorescence spots indicating the presence of unreacted AFB1 and its derivatives.

4.2.2. Evaluation of AFB1-lys and AFB1-oval adducts

ELISA performed to evaluate the AFB1-lys and AFB1-ova adducts as detailed in 3.6. The detection limit of AFB1-lysine for antiserum was found to be 7.8 pg ml^{-1}. Therefore, for competitive ELISA AFB1-lys standard was used from 2000 to 7.8 pg ml^{-1} as working concentration. At this concentration standards gave regression of 0.994 (from three experiments). The regression curve for AFB1-lys was shown in Fig 17.

To determine the AFB1-ova adduct concentration, it was tested from 100 ng - 0.78 ng ml^{-1} in ELISA. After 10.0 ng ml^{-1} there was no variation in absorbance values measured at 405 nm. AFB1-ova concentration of 10 ng ml^{-1} was found to be best suited for competitive ELISA.

Figure 13. Spectrophotometric graph of AFB1-lys adduct taken from 200-400 nm. This shows one peak at 275 nm and the other one at 340nm.

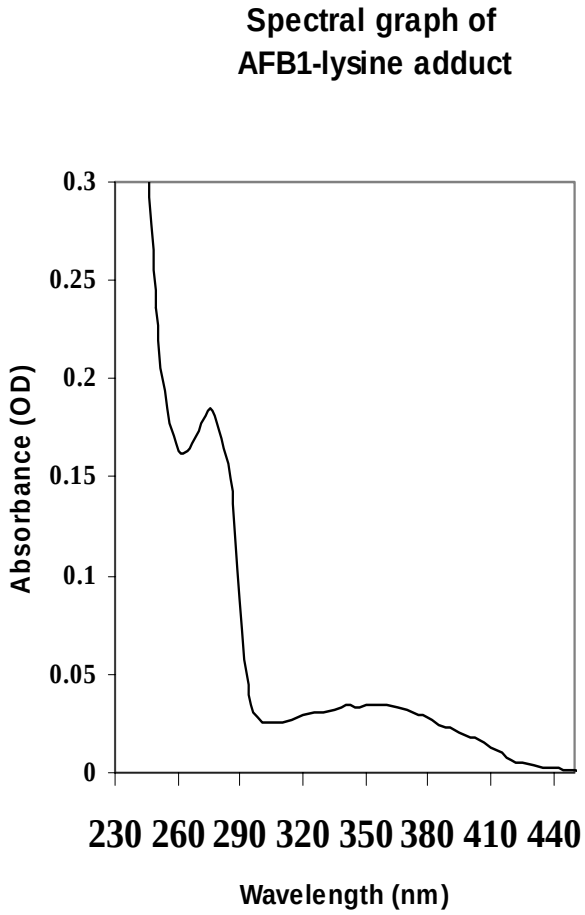


Figure 14. Spectrophotometric graph of AFB1-ova from 200-400 nm shows Note two peaks, one at 275 nm and the other one at 365 nm with multiple minor peaks.

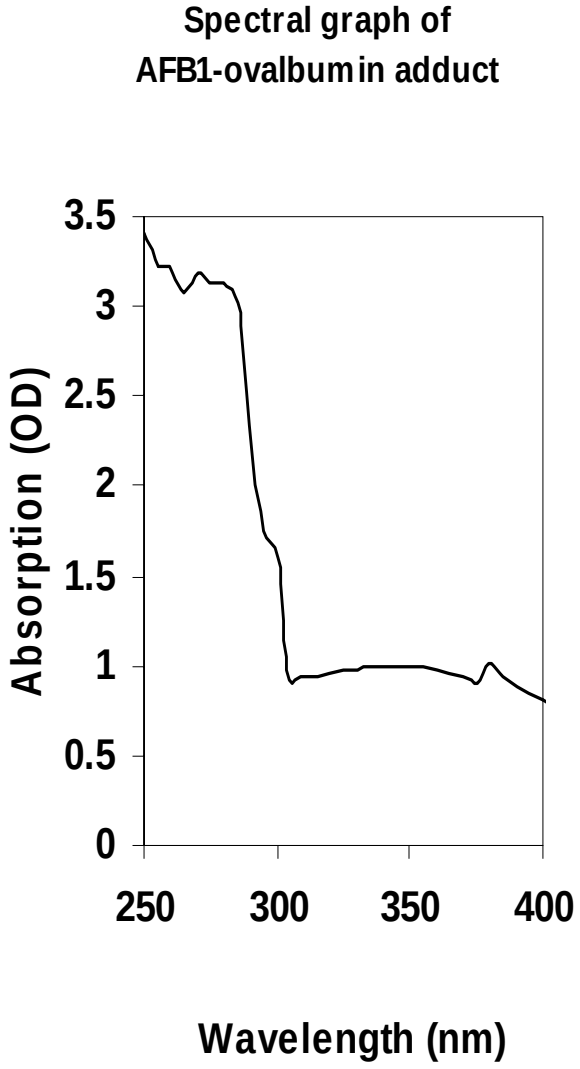
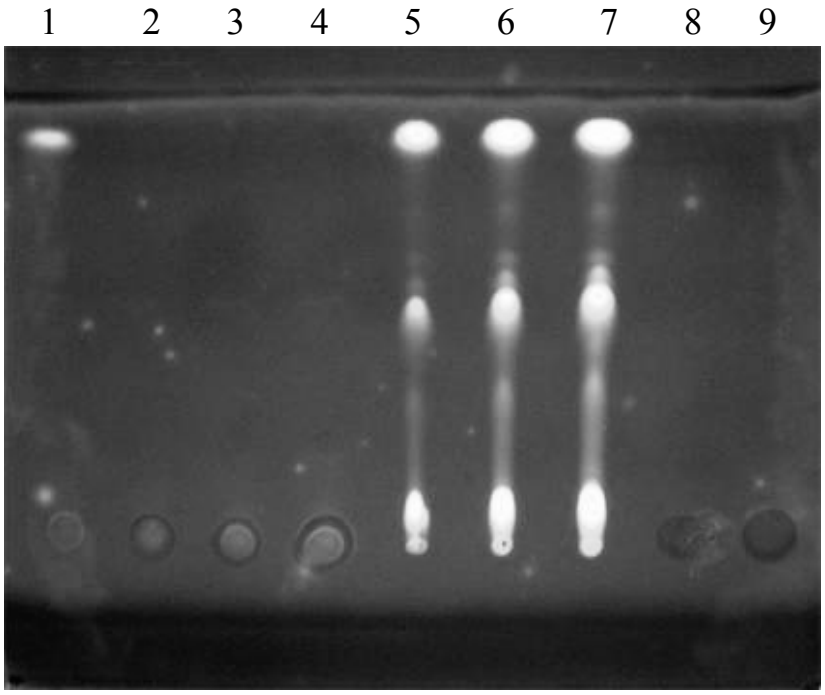


Figure 15. TLC to evaluate AFB1-lysine adduct formation

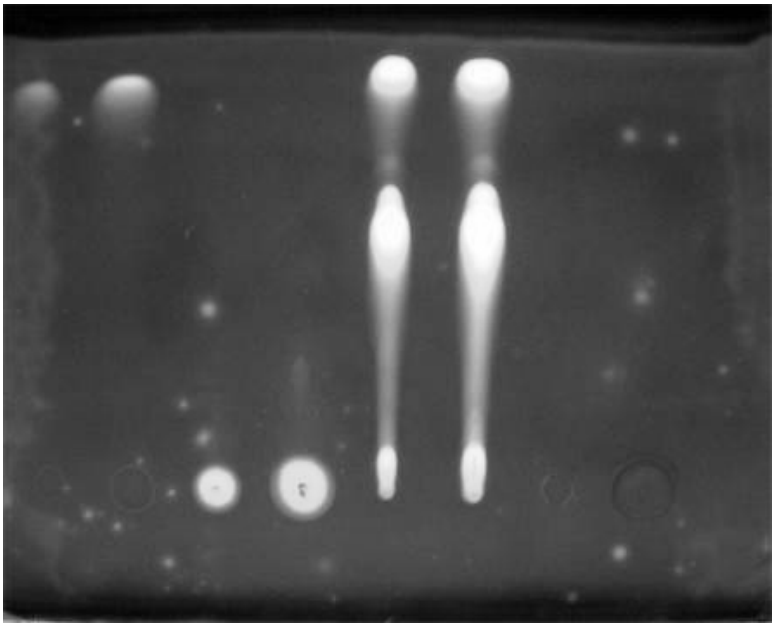


Lane 1, AFB1 standard 20 µl
Lane 2, AFB1 lysine 20 µl
Lane 3, AFB1 lysine 40 µl
Lane 4, AFB1 lysine 60 µl
Lane 5, DCM fraction 20 µl
Lane 6, DCM fraction 40 µl
Lane 7, DCM fraction 60 µl
Lane 8, Lysine 10 µl
Lane 9, Lysine 20 µl l

Note that, AFB1 fluoresces under UV light. AFB1-lys adduct has no relative mobility and therefore the fluorescence appears at the spot (lanes 2, 3 and 4); lysine lacks fluorescence (lanes 8 and 9); and multiple spots in organic phase (lanes 5, 6 and 7) suggests the partial reactants, unreacted AFB1.

Figure 16. TLC to evaluate formation of AFB1-ovalbumin adduct

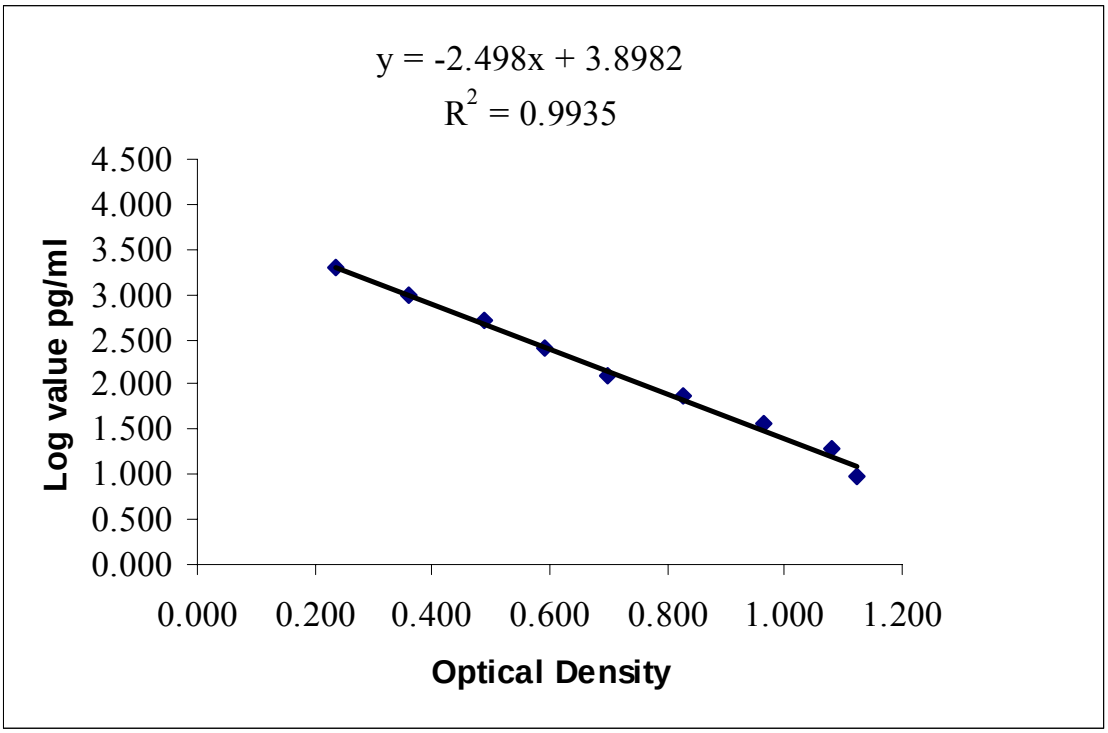
1 2 3 4 5 6 7 8



Lane 1. AfB1 standard 10 µl
Lane 2. AfB1 standard 20 µl
Lane 3. AfB1-oval adduct 10 µl
Lane 4. AfB1-Oval adduct 10 µl
Lane 5. Organic phase 10 µl
Lane 6. Organic phase 20 µl
Lane 7. Oval standard 10 µl
Lane 8. Oval standard 20 µl

Note that AFB1-ova adduct has no relative mobility and therefore the fluorescence appears at the spot (lanes 3 and 4); ovalbumin lack of fluorescence (lanes 7 and 8); and multiple spots in organic phase (lanes 6 and 7) suggests the partial reactants, unreacted AFB1.

Figure 17 Regression curve plotted using ELISA absorbance values of AFB1-lys standards measured at 405 nm



4.2.3. Validation of IC-ELISA using HPLC

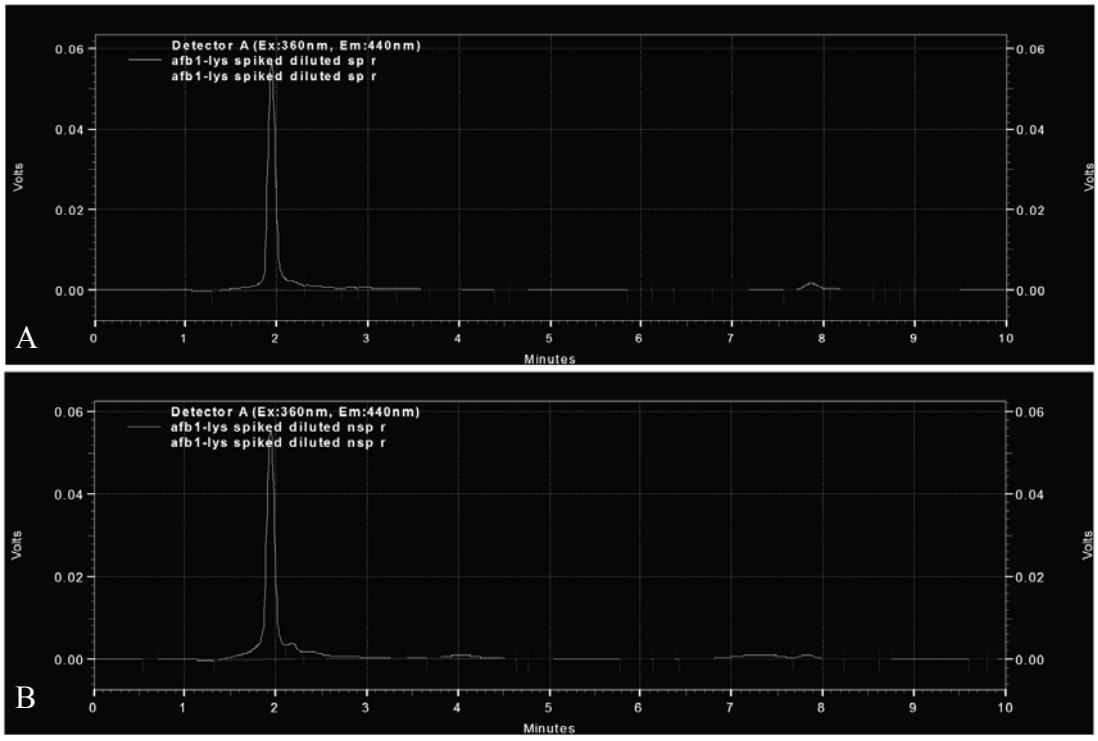
Results of IC-ELISA were compared with HPLC carried out using reversed-phase C18 column. Two peaks were obtained in HPLC, one major peak at 2.0 min corresponding to AFB_1-lys and one minor peak at 2.4 min (Fig 18), suggesting that AFB_1-lys had a relative retention time of 2 min. Each fraction was collected and dried in a vacuum evaporator and sample was reconstituted with 250 µl of PBS and analyzed in IC-ELISA. Results revealed high correlation in AFB_1-lys estimates by HPLC and IC-ELISA (SD±1.7 to 17.8) (Table 6). HPLC analysis of albumin samples hydrolyzed and purified with Sep-Pak cartridge (Fig 18A) or ethanol (Fig 18B) gave similar results (Table 6).

Table 6. Quantitative estimation of AFB_1-lys in human serum albumin by HPLC and in HPLC fraction by IC-ELISA

Sample No.	Concentration of AFB1-lys (pg/mg)		% recovery of AFB_1-lys in HPLC fraction by IC-ELISA
	HPLC	**IC-ELISA**	
1[a]	72	69.53	96.5 (±1.7)
1[b]	72	67.2	93 (±3.4)
2[b]	109.08	103	94.2 (±4.3)
3[b]	101	75.82	75.0 (±17.8
4[b] (negative control)	0[c]	-[c]	-[c]

[a]Hydrolyzed sample purified with Sep-Pak cartridge; [a]Hydrolyzed sample purified by ethanol precipitation; [C]No peak in HPLC; OD equivalent to negative control in ELISA)

Figure 18. Reversed phase HPLC of proteinase-K hydrolyzed human serum albumin spiked with AFB_1-lys. Sample purified using Sep-Pak cartridge (A) and purified with ethanol precipitation (B).



4.3 Sample analysis

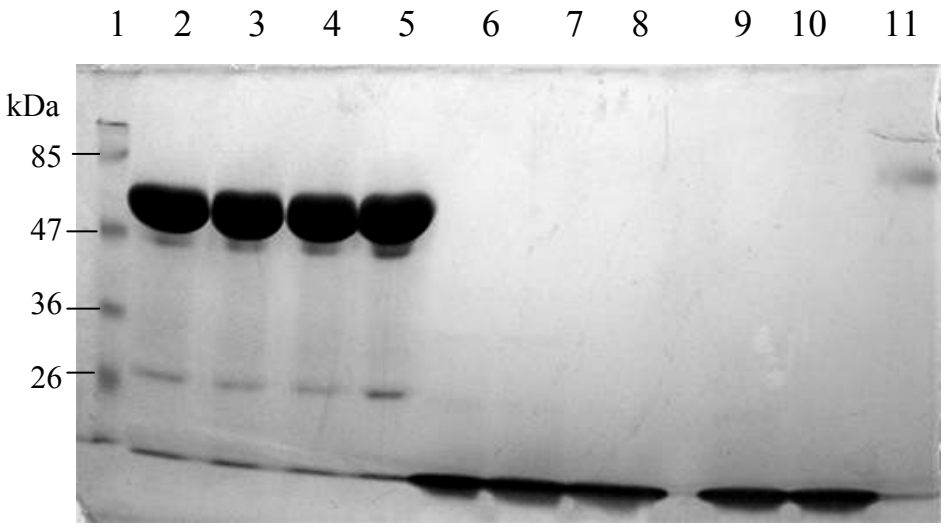
4.3.1. Albumin hydrolysis

The concentration of the albumin obtained was approximately 12 mg 500 μl^{-1} of serum. Figure 19 shows the purity of albumin extracted from serum and it also shows the complete digestion of albumin with proteinase K enzyme. The Lane 1 shows Protein standards (MBI Fermentas, Cat# SM0441), Lanes 2-5 shows albumin extracted from human serum, Lane 6 shows Albumin artificially spiked with AFB1-lys and hydrolyzed with proteinase K, Lane 7-10 shows albumin hydrolyzed with proteinase K and Lane 11, shows Proteinase K.

4.3.2. Testing of recovery of AFB1-lysine in artificially spiked albumin sample

The artificial spiking experiment revealed 96±11% recovery of the AFB_1-lys in IC-ELISA (Table 7). Comparison of efficacy of albumin hydrolysis using ethanol precipitation procedure and Sep-Pak cartridges found no significant differences, indicating that low-cost ethanol precipitation approach is effective (Table 8).

Figure 19. Separation of purified albumin and hydrolyzed albumin in 12% SDS-PAGE and the gel was stained with silver nitrate to visualize the proteins



Lane 1, Protein standards (MBI Fermentas, Cat# SM0441)

Lanes 2-5, Albumin extracted from human serum

Lane 6, Albumin artificially spiked with AFB1-lys and hydrolyzed with proteinase K

Lane 7-10, Albumin hydrolyzed with proteinase K

Lane 11, Proteinase K

4.3.3. Indirect competitive ELISA for estimating the concentration of AFB1-lysine adduct in serum samples

IC-ELISA developed shows the regression of 0.99 for the standards (7.8 to 2000 pg ml^{-1}) (Fig. 17). The AFB1-lysine concentration in samples was expressed in pg mg^{-1} of albumin in positive samples with mean aflatoxin-albumin adduct level of 86 samples are 181.9 ± 5 and SD 1.49 ± 7. AFB_1-lys was detected in 86 samples at a concentration between 2.5 to 677 pg mg^{-1} albumin (Table 9; Table 11; Master chart 1). All the samples that were positive to AFB_1-lys were from HBV positive subjects, indicating a potential risk of HCC in 12.7% of the 673 subjects tested in this study.

Mean aflatoxin-albumin level according to gender, HBV infection, socioeconomic status and age is given in Table 10. The presence of hepatitis B and aflatoxin B1 together increases the severity of liver diseases which is represented by pb value 0.039 (<0.05). The mean afltoxinB1-albumin adducts level in hepatitis B positive patients are 202.0 with 95% confidence interval of (158.4-245.7). Where as the mean adduct level in control samples are 105.8 with 95% confidence interval of (35.04-176.6). These control samples are collected from the same family members who consume same lot of food but they clinically don't have any symptom of liver disease at the time of collection of samples. There is no significant difference in mean aflatoxin-albumin level according to age, socioeconomic status and gender.

Table 7. Percentage recovery of AFB_1-lys from spiked human serum albumin by IC-ELISA

Sample no	AFB_1-lys spiked in human serum albumin (pg)	Concentration estimated by IC-ELISA (pg/mg)		% recovery*		
		Expt.1	Expt. 2	Expt. 1	Expt. 2	SD (±)
1	2000	1677	1832	83.8	91.6	5.5
2	1000	1049	979	104.9	97.9	4.9
3	500	556	483	111.2	96.6	10.3
4	200	189	212	94.5	106	8.1
5	50	51.38	52.85	102.7	105.7	2.1

*(Concentration estimated by IC-ELISA/amount of AFB1-lys spiked) × 100

Table 8 Effect of hydrolyzed albumin purification method on AFB_1-lys detection in IC-ELISA.

Sample No.	Concentration of AFB_1-lys spiked in HSA (pg/mg)	AFB_1-lys recovered (pg/mg)*	
		Sep-Pak cartridge[a]	Ethanol purification[a]
1	0	-[b]	-
2	0	-	-
3	0	-	-
4	0	-	-
5	50	46.12 (92%)	47 (94%)
6	200	189.76 (94%)	212.50 (106%)
7	1000	1038.5 (103%)	1049.29 (104%)

*HSA=human serum albumin (Sigma).
[a]Method used for purification after albumin hydrolysis. [b]OD values ≤negative control.0

Table 9. Estimation of AFB_1-lys in human serum albumin by IC-ELISA

AFB_1-lys adduct concentration (pg/mg albumin)[a]	Number of samples
-[b]	587
<100	38
100-500	42
500-1000	6
Total	673

[a]Albumin purified from serum fraction was hydrolyzed with proteinase-K, purified by ethanol and tested in IC-ELISA in duplicates. [b]OD equivalent to negative control in ELISA

Table 10. Mean afaltoxin B1-albumin according to gender, HBV infection and socioeconomic status.

Parameter	Mean aflatoxin level	p value	95% CI
Gender			
Female	164.8	0.602	88.9-240.7
Male	187.8		142.9-232.7
Hepatitis B			
Negative	105.8	0.039	35.04-176.6
Positive	202.0		158.4-245.7
Socio economic status			
Poor	150.8		101.7-199.9
Middle	206.5	0.15	150.2-262.8
Rich	-		
Habits			
Alcoholic	140.9	0.44	53.89-227.9
Non-alcoholic	187.3		145.5-229.1
Age			
35-40	122.25		
40-45	173.51		
45-50	218.18		
50-55	145.19		
55-60	174.09	0.69	

Table 11. Concentration of AFB1-lysine pg mg^{-1} albumin

S.No	Concentration of AFB1-lysine pg mg^{-1} albumin (values from two experiment)	Standard Deviation
1	31.5	0.7
2	129	0
3	111	1.4
4	676.5	4.9
5	403.9	5.6
6	180.8	1.2
7	249.1	5.3
8	393.4	4.9
9	153.1	0.2
10	208.4	0.7
11	68.7	0.9
12	597.5	3.5
13	164.6	0.4
14	318.2	2.4
15	54.5	2.1
16	26	2.8
17	58.5	2.1
18	27.5	2.1
19	32.9	1.5
20	115.1	1.2
21	186.2	5.3
22	30.7	1.0
23	121.4	2.1
24	73.3	6.6
25	83.7	7.4
26	119.4	0.8
27	169.7	7.4
28	129.5	0.7
29	114.2	1.0
30	132	4.2
31	57.5	0.7
32	38	0
33	110	0
34	33.75	3.1
35	185.85	5.8
36	43.1	4.1
37	118.4	0.6
38	250.6	7.5
39	116.0	5.5
40	547	4.2
41	466.5	3.5
42	117.1	4.0
43	419.5	9.1
44	394.5	7.7

S.No	Concentration of AFB1-lysine pg mg^{-1} albumin (values from two experiment)	Standard Deviation
45	257	4.2
46	438	0
47	27.5	2.1
48	33	4.2
49	330	7.0
50	121.5	4.9
51	603.5	4.9
52	286	0
53	42	0
54	229	1.41
55	19.5	0.7
56	343	2.8
57	36.5	0.7
58	450	0
59	79.5	3.5
60	25.5	0.7
61	57.5	2.1
62	29.5	0.7
63	76	4.2
64	438	2.8
65	622.5	3.5
66	283.5	4.9
67	617.5	3.5
68	234	2.8
69	322.5	3.5
70	41	0
71	52.5	0.7
72	74.1	1.4
73	2.75	0.2
74	593.5	0.7
75	41	0
76	25	0
77	49	0
78	14	1.0
79	9.1	0.2
80	77.2	0.3
81	34.5	0.7
82	114.6	0.5
83	112.5	0.5
84	91.7	0.3
85	90.5	0.6
86	159.6	3.2
	181.9*	1.49††

***Mean AFB1-lysine**
††Mean standard deviation

4.4 Production of monoclonal antibodies

4.4.1. Immunization

The immunization protocol followed in this experiment was good enough to elicit the immune response. The sera from mice immunized with 21 days protocol showed good antibody response. The mice selected for fusion showed AFB1-KLH polyclonal antibody titer 1:10,000.

4.4.2. Fusion and cloning

The fusion efficiency (number of wells showing cell multiplication in each well of 96-well plate) was 80%. The cells derived from 15 wells showed specific binding to Aflatoxin-BSA adduct. But after two successive transfers only one retained the antibody activity. The growing hybridoma cells are shown in fig 20.

4.4.3. Titer and characterization of antibody

The titer of antibody was found to be 1: 5000. The antibody was highly specific for AFB1-BSA adduct. There was absolutely no cross reaction with BSA,Ovalbumin and Human serum albumin. The designation, cross reactivity and minimal inhibition values for monoclonal antibody (IG8) that showed high specificity to AFB1-BSA is presented in Table 12. It has the detection range from 1 μg to 4 ng ml^{-1} with 50% inhibition at 0.13 μg ml^{-1}. The dose response curve of AFB1-BSA is given in Fig 21.

Table 12. Cross reaction and minimal inhibition value of clone 1G8

Designation	Cross reaction (%)					Minimum inhibition (μgml^{-1})
	AFB1-lysine	AFB1-BSA	BSA	Ovalbumin	HSA	AFB1-lysine
1G8	100	100	0	0	0	0.004

Figure 20. Growing hybridoma cells produced against AFB1-KLH conjugate on 7^{th} day.

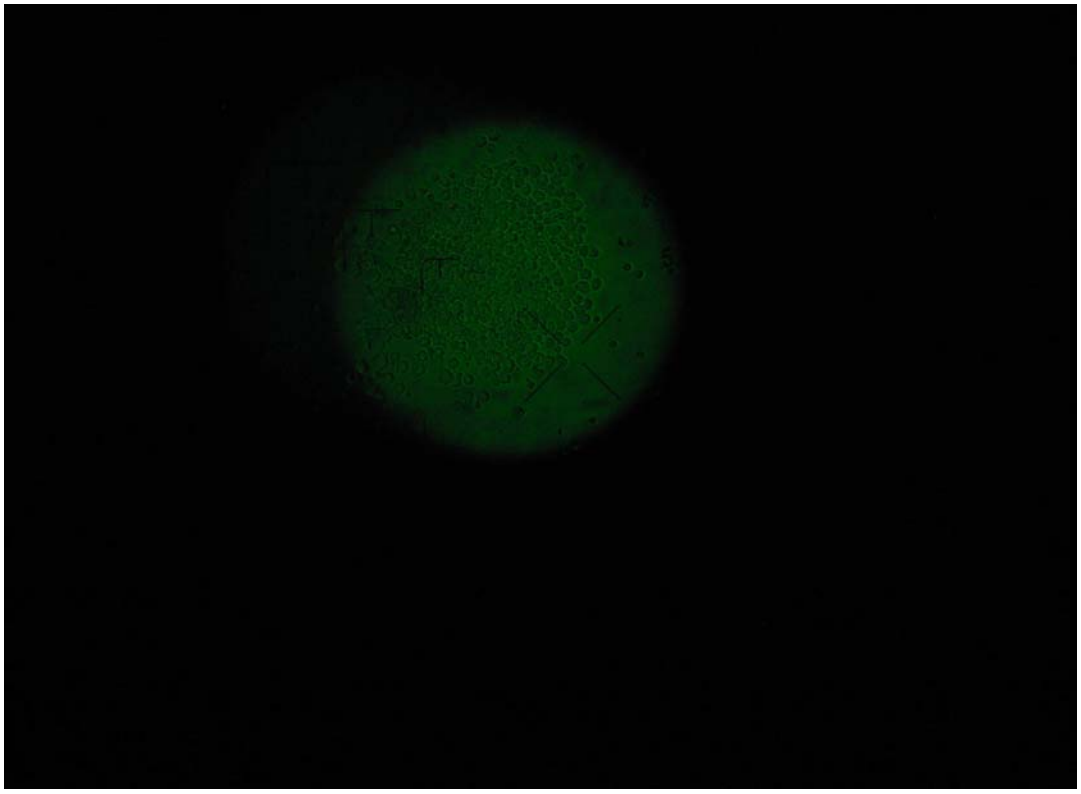
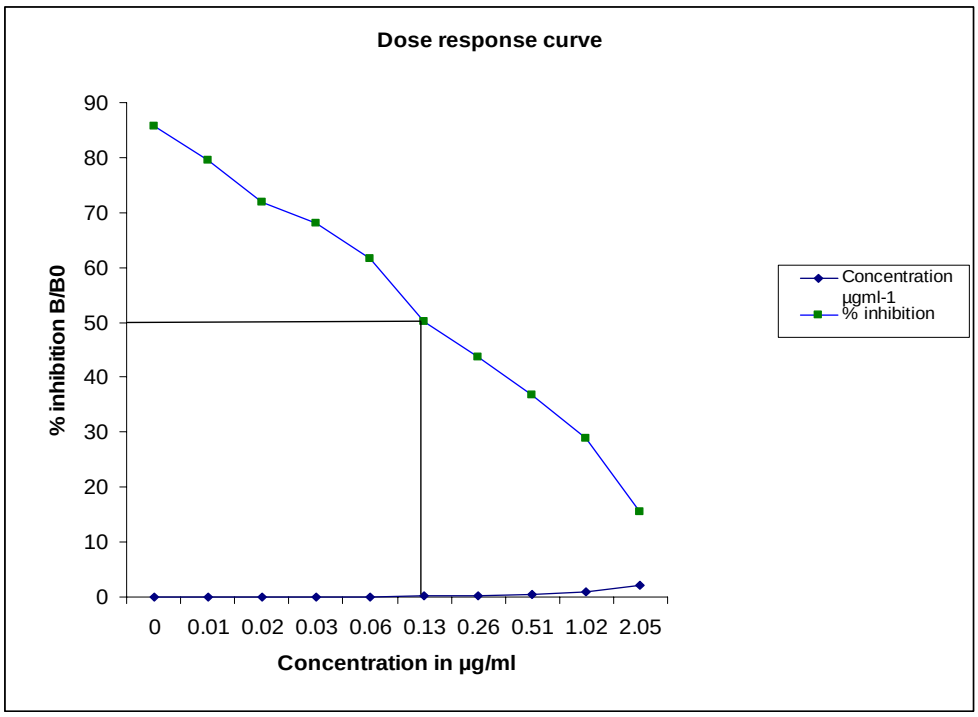


Figure 21. Dose response curve of aflatoxinB1-BSA. Fifty percent inhibition value of AFB1-BSA was 0.13 µg ml^{-1}.



4.5 Detection of *p53* Mutation using Restriction Fragment Length Polymorphism (RFLP)

4.5.1. DNA extraction

DNA was extracted from whole blood using neucleospin kit as detailed in the Section 3.5.3. This resulted in good quality DNA from all 238 samples. The genomic DNA was used for PCR amplification as detailed in the Section 3.5.5, using the forward and reverse primers which yielded 254 bp amplicon (Fig 22).

4.5.2. Restriction Fragment Length Polymorphism (RFLP)

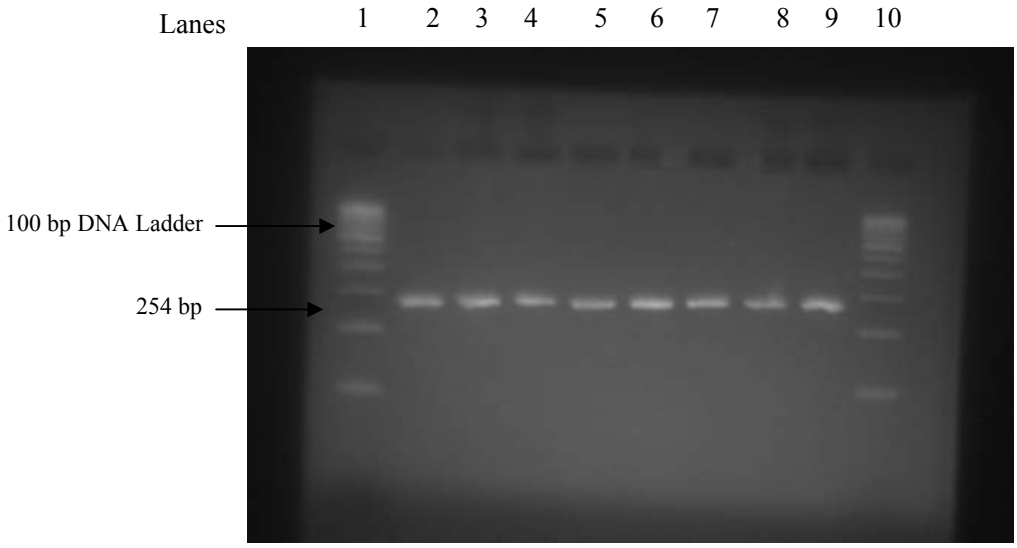
The RFLP result indicates that the absence of mutation at codon 249 results in 92 bp, 66 bp and several small fragments; whereas mutation at the codon 249 eliminates the *Hae* III restriction site resulting in undigested amplicon and 158 bp fragment (Fig 23).

A totally of 238 samples were analyzed for *p53* gene mutation. Subject age ranged from 36 to 61 years, 64% of them were men, 130 were *Hepatitis B virus* (HBV) positive and 108 were negative to HBV. Among 130 HBV positive samples 112 samples are having cirrhosis of liver. The *p53* mutation was detected in 10 (4.2%) samples, 8 of which were from HBV positive subjects and also positive for aflatoxin-albumin adduct ($p = 0.07$); Remaining two were from HBV negative subjects, but positive to aflatoxin-albumin adduct ($p = 0.14$).

In total 37 of 238 samples analyzed tested positive to aflatoxin-albumin adduct in IC-ELISA. The concentration of aflatoxin adduct ranged from 2.5 to 677 pg mg^{-1} of albumin (Master chart 2). Among these, 29 samples were HBV positive and 8 samples were positive to both HBV and *p53* mutation ($p=0.07$). The Chart 1 shows the relationship between *p53* mutation and aflatoxin-albumin positivity. At 10% level of significance if probability <0.1 then presence of aflatoxin-albumin adduct and *p53* mutation are dependent, otherwise independent (Table 13; Fig 24). This shows that *p53* mutation and aflatoxin-albumin adduct positivity are dependent in HBV samples. We

found there was no significance in the 249ser mutation between females with HBV (p=0.12) and males with HBV (p=0.38). This shows gender has no influence on aflatoxin induced *p53* mutation.

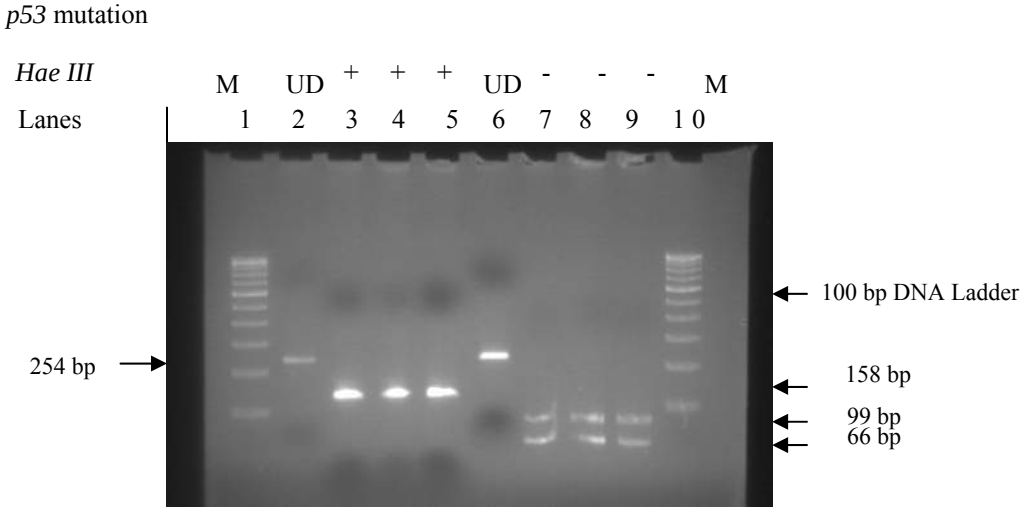
Figure 22. The electrophoresis map of PCR products



Representative ethidium bromide stained 2 percent agarose gel containing undigested PCR products (lanes 2-9), Lane 1and 10 shows 100bp DNA ladder, which was used as a size marker.

107

Figure 23. The electrophoresis map of RFLP



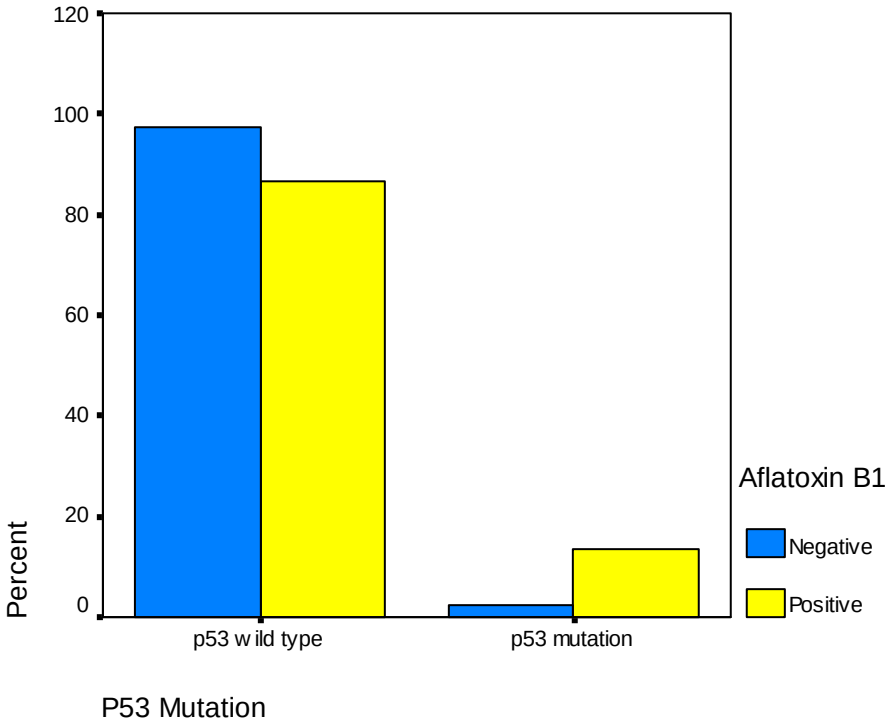
Representative ethidium bromide stained 2 percent agarose gel containing undigested PCR products (lanes 2and 6), PCR products digested with *Hae III* (lanes 3,4,5,7,8 and 9) ,lane 3,4 and 5 shows *p53* mutation, lane 7,8 and 9 shows wild type. Lane 1and 10 shows 100bp DNA ladder, which was used as a size marker.

Table 13. Demographic Data on Patients with hepatitis B

Parameter	No of subjects analyzed (n=238)	*p53* mutation (%)	Aflatoxin-lys (%)	p value
Serum Hepatitis B				
Positive	130 (54.6)	8(6.1)	29(22.0%)	0.14
Negative	108 (45.4)	2(1.8)	8(7.4%)	0.07
Sex				
Male				
Hepatitis B Positive	88(57.5)	7(7.9)	24(27.2)	0.38
Hepatitis B Negative	65(42.4)	2(3)	7(10.7)	0.21
Female				
Hepatitis B Positive	42(49.4)	1(1.17)	5(5.8)	0.12
Hepatitis B Negative	43(50.5)	0(0)	1(1.17)	--

Note: ' - ' means Row or column sum zero. No statistics is computed.

Figure 24 comparative studies of *p53* mutation and aflatoxin B1 in case and control samples.



5. DISCUSSION

5. Discussion

Many crops and commodities are vulnerable to attack by a group of fungi that are able to produce toxic metabolites called mycotoxins. There are three major genera of fungi that produce mycotoxins: *Aspergillus, Fusarium* and *Penicilium*. Of these, toxins produced by *A. flavus* and related species are most potent. They are referred to as aflatoxins. Several types of aflatoxins occur in nature. Of these, AFB1 has been recognized as the most potent toxin. It has been classified as a carcinogen. They enter into human and animal systems through consumption of contaminated food. Although food safety regulations have been established in most countries to regulate the levels of toxin within prescribed limits in food and feed, the implementation of these regulations is poor in developing countries. As a consequence, populations, especially from economically poorer sections, are most vulnerable to aflatoxin exposure. Thus, AFB1 poses a serious health threat to individuals in many underdeveloped countrie where this mycotoxin is present in staple foods (Detroy *et al*., 1971; Groopman *et al*., 1988).

Most of the interest in AFB1 has been in the area of liver cancer and genotoxicity (Busby and Wogan, 1984; Groopman *et al*., 1988). The major reaction in AFB1-metabolism is relatively well established (Fig. 2). CYP450 oxidizes AFB1 to AFB1-8,9 endo and exo epoxide. Only the exo epoxide can bind to DNA and protein (Guengerich *et al*., 2002). Epidemiological studies provide strong evidence of an association between chronic HBV infection and primary hepatocellular carcinoma (Beasley *et al.,* 1981; Beasley, 1987). However, other types of exposure are known to play a role in the pathogenesis of liver cancer. In populations with a high prevalence of HBV infection, a relationship has been observed between mortality from primary hepatocellular carcinoma and dietary AFB1 exposure (Van Rensburg *et al*., 1985; Peers *et al.,* 1987; Yeh *et al.,* 1989). It is not yet clear whether AFB1 exerts its carcinogenic effect in individuals in the absence of chronic infection with HBV (Wild *et al.,* 1990). However, AFB1 has been shown to be a carcinogen in many species. Human liver cells have been shown to be capable of metabolizing AFB1 *in vitro* to the biologically reactive 8,9-epoxide, which can form adducts with DNA and protein, and thereby results in DNA damage.

Recently, several method have been developed to measure individual levels of exposure to aflatoxins. The information provides data on risk posed due to aflatoxins. It has been demonstrated that AFB1 binds covalently to albumin. AFB1-lysine has been identified as a major adduct in rat albumin. It has also been observed in human sera (Wild *et al.,* 1986; Sabbioni *et al.,* 1987; Wild *et al.,* 1990; Anitha *et al.,* 2011). In experimental animals, the level of albumin adduct reflects the degree of DNA damage in the liver (Turner *et al.,* 1998). Thus, AFB1-lys acts as biomarker for assesses sent of human exposure to aflatoxins.

Aflatoxin albumin adducts are found in peripheral blood after exposure to aflatoxin B1. The measurement of these adducts is a potentially useful tool in the epidemiological studies of the role of AFB1 in the etiology of liver cancer. Three complementary approaches to the quantitation of AFB1-albumin adducts are described: (a) Enzyme linked Immunosorbent assay (ELISA) performed directly on intact albumin (direct ELISA); (b) ELISA performed on an albumin hydrolysate (hydrolysis ELISA); (c) high performance liquid chromatography (HPLC) fluorescent detection of AFB-lys adduct after albumin hydrolysis and immunoaffinity purification. These techniques have been validated by direct comparison with rat albumin samples modified to a known extent *(reference)*. Detection limits of 100, 50 and 5 pg, aflatoxin per mg human albumin were determined for the three methods respectively (Turner *et al.,* 1998). Samples obtained from individuals from Thailand, the Gambia, Kenya, and France have been used to validate the measurement of AF-albumin adducts by these three methods (Turner *et al.,* 1998). Levels of 5 to 1064 pg aflatoxin-albumin adduct mg^{-1} of albumin were observed in the former two countries while no adducts were detected in samples from France (Turner *et al.,* 1998). The relative properties of the three assays, with special regard to their application in epidemiological studies, are considered to detect aflatoxin in human (Wild *et al.,* 1990). A combination of the hydrolysis ELISA for large scale screening followed by confirmatory analysis in positive samples by HPLC is suggested as an optimum methodology (Wlid *et al.,* 1990; Anitha *et al.,* 2011).

ELISA was used in earlier studies to determine human exposure to aflatoxin at an individual level and consequently allows a better assessment of the role of aflatoxin and its interaction with hepatitis B virus infection, in the aetiology of liver cancer. Measurements of aflatoxin bound to serum albumin in children and adults from various African countries shows that between 12 and 100 % contain aflatoxin-albumin adducts, with levels up to 350 pg AFB1-lysine equivalent/mg albumin. In Thailand, lower levels and prevalence of this adduct were observed, while no positive sera were detected from France or Poland (Wild *et al.,* 1990). The study in 250 samples in India showed aflatoxin-albumin adducts level ranged from 2.5 to 75 pg mg^{-1} albumin (Anitha *et al.,* 2011).

Studies on AFB1 exposure in the United Kingdom population were done by measuring levels of serum AFB1-albumin, using ELISA and high-performance liquid chromatography with fluorescence detection. Serum albumin was extracted, and AFB1-lysine, the digest product of AFB1-alb, was isolated and measured. A good correlation was found between calibration of ELISA results and HPLC ($r=0.972$; $p<0.001$). ELISA was subsequently used to analyze human serum albumin (Turner *et al.,* 1998). Thus this study demonstrated that ELISA is sensitive in detection of AFB1-lys biomarkers and results are comparable to HPCL studies.

Therefore, to assess the level of aflatoxin exposure among populations in India, this study was undertaken to standardize the methods for the detection of aflatoxin biomarker by ELISA using antibodies produced at ICRISAT and it is validated (Anitha *et al.,* 2011) with HPLC as described by (Sabbioni, 1990).

Earlier, AFB1-lysine and AFB1-ovalbumin adducts were prepared *in vitro* using AFB1-dibromide (Sabbioni *et al.,* 1987; Chapot and Wild, 1991). This was a tedious method to prepare adduct and was time consuming. In this study, AFB1-lysine and AFB1-ovalbumin was prepared by oxidizing the AFB1 to AFB1-8,9 epoxide by the chemical oxidant MCPBA. Long incubation periods of up to 6 h can form more epoxide when compare to short incubation period. The epoxide was then conjugated with N-α-acetyl lysine or ovalbumin through overnight incubation. Since the ovalbumin does not

dissolve completely, longer incubation time with gentle shaking may permit the formation of more conjugate.

The spectral reading of AFB1-lysine gave two peaks at 275 nm and 340 nm. In the study of Sabbioni (1990) AFB1-lys peaks were at 275 nm and 335 nm. In this study, the peaks are near enough (275nm and 340nm). But the difference between absorption value between 335 nm and 340 nm was found to be only 0.1 OD. The adduct was aliquoted and stored at –20˚C. It was stable for over a year. Sabioni and Sepai (1998) found that the adduct can be stored over a year as solid or in solution at pH 7.4 to 8.0 at –50˚C. In present study, when adduct was stored at 4˚C in liquid form. There was a 20% loss within two months.

Albumin prepared from the serum by the protocol detailed in section 3.3.4 gave high yield and purity. The yield was Approximately 12 mg 500 μl^{-1} of the albumin when saturated ammonium sulphate (4.1M), which has neutral pH, was used. Wild *et al.,* (1990b) got approximately 10 mg 500 μl^{-1}. It was about 50% of the theoretical yield. For ELISA, less than 2 mg was deemed to be sufficient.

Since AFB1 is a small molecule, it does not elicit good immune response in animals. Therefore, it is conjugated to a hapten or carrier protein (Waliyar *et al*., 2005a). Usually BSA is used as carrier protein for immunizing rabbits (for polyclonal antibodies or mice, for monoclonal antibodies). Since in human and animals, AFB1 occur as an epoxide, the AFB1 molecules are first modified to epoxide using chemical oxidation. This is linked with BSA and used for immunization. Similar protocol was used in this study to produce antibodies to AFB1 epoxide (Srinivas, 2003). These antibodies were then utilized in this study to detect AFB1-lys in human serum. It was validated using HPLC (Anitha *et al.,* 2011). Since antibodies produced against AFB1 epoxide may cross react with pure AFB1, comparative studies were done using antisera to AFB1 and AFB1 epoxide. This showed that antibodies produced to AFB1 epoxide can cross react with AFB1, but antibodies to AFB1 did not react with the epoxide form. To eliminate any cross reaction with albumin, antibodies were adsorbed with human serum albumin prior

to use in ELISA. Further, serum albumin from healthy controls was used in AFB1-lys standards to minimize the cross reaction affect on the final data, which are deduced from the standard graph plotted from AFB1-lys standards. Wild *et al.* (1990) used human serum albumin from healthy controls supplied by Sigma.

The detection limit of AFB1-lysine for the ICRISAT antisera was found to be 7.8 pg ml^{-1} to 2 ng ml^{-1} (Anitha *et al.,* 2011). Turner *et al*. (1998) used 7.13 pg ml^{-1} to 1.83 ng ml^{-1} for ELISA.

Monoclonal antibody

The monoclonal antibody that was produced against AFB1-KLH is highly specific to AFB1-albumin. Since the conjugate is produced by epoxidation process it can identify any AFB1-epoxide conjugates. However, the monoclonal antibody had a detection limit of 4 ng per ml-1. Whereas, the polyclonal antibody had a detection limit of 7.8 pg ml-1. Hence, the polyclonal antibodies were considered more suitable for the detection of afaltoxin-albumin adduct in human blood.

Sample processing

Albumin hydrolysis was carried out with proteinase K. Proteinase K digestion results in breakdown of proteins into individual amino acids and oligomers of short size. Therefore, albumin digested with proteinase K was recommended for purification through columns to remove undigested albumin and large sized peptide. But, in this study we found that albumin hydrolyzed with proteinase K for longer time (17 h and more) did not contribute any background and thus the column clean up of the sample was eliminated further simplifying the procedure. This reflected in 96±11% recovery of the AFB1-lysine in artificially spiked samples, suggesting that there was not much loss during digestion with proteinase K.

In this study, HBV positive samples and healthy control were used to validate the protocol for standardization of assessment of AFB1-lys in serum. Of 673 samples, 86 samples tested positive to AFB1-lys, indicating exposure to aflatoxins. In these 86

samples which showed aflatoxin contamination, the aflatoxinB1-albumin content ranged from 2.5-677 pg mg^{-1} (mean adduct level is 181.9 ±5 and SD 1.49 ± 7.). With the standardized protocol, large numbers of samples could be analyzed to determine aflatoxin exposure in humans.

At 5% level of significance p value 0.039 (<0.05) indicates presence of aflatoxinB1-lysine adducts and hepatitis B has synergistic effect on liver damage. The hypothesis of current experiment is aflatoxin B1 and hepatitis B together aggravates the liver damage. When liver is exposed to Hepatitis B virus the hepatocytes get cell injury and undergo structural changes (chapot and wild 1991). At the same time the entry of aflatoxin into the hepatocyte increase the degree of liver cell damage. The aflatoxins which get accumulated in liver cells will bind with plasma protein albumin which is basically produced from liver cells. Apart from this when the hepatocytes multiply during repair process the aflatoxins bind to DNA and leads to mutation. The result in this study also shows that the presence of hepatitis B activates aflatoxin B1 which then together damages the liver cells rather than aflatoxin B1 alone or Hepatitis B alone. These results are similar to that of Kew, 2003.

In a previous Indian study Murugavel et al (2007)using immunoperoxidase test, reported association of AFB_1 deposits in 58% of 32 human liver biopsies samples from HCC cases, 15 of which were positive to HBV. However, in the same study ELISA assay for AFB_1 biomarker was reported negative. A reason for this disparity is unclear. However, it is speculated that this may be linked to time of assessment, as AFB_1-lys biomarker is detectable for up to 2 months from the first exposure to aflatoxin. Nonetheless, Murugavel et al., demonstrated significant association of AFB_1 toxicity with HCC cases in India, and emphasize the need for wider surveillance to determine AFB_1 exposed populations in India.

The IC-ELISA developed in this study could be a potential tool for such epidemiological studies to identify vulnerable groups. This test is simple to perform, cost-effective and enables high-throughput analysis. After thorough validation, IC-ELISA has

the potential to serve as a rapid tool to identify AFB_1 exposed individuals. Vulnerable cases can be subjected to further specific tests to assess HCC risk, institute remedial treatments and implement appropriate strategies to minimize aflatoxin contamination in diets of communities at high risk of AFB_1 exposure.

Aflatoxins together with chronic hepatitis B virus (HBV) infection contribute to the high incidence of hepatocellular carcinoma in developing countries (Williams *et al.,* 2004). An understanding of the mechanism of interaction between these two factors could provide a strong rationale for developing effective prevention strategies. A study in the Gambia, the effect of environmental factors (place of residence and timing of sample collection) and host factors (age, sex, HBV status and inter-individual variations in carcinogen metabolizing enzymes) in determining blood-aflatoxin adduct levels in 357 individuals, of whom 181 were chronic HBV carriers, emphasizes the priority to reduce aflatoxin exposure in these communities by primary prevention measures (Wild *et al.,* 2000).

Usually, people avoid moldy foods and humans are usually an aflatoxin tolerant species. However, in times of food scarcity, or under conditions of poverty, people usually have no option but to use lower-priced, poor quality food, which is commonly contaminated (Williams *et al.,* 2004). Post harvest measures to restrict aflatoxin contamination of groundnut crops were shown to reduce exposure in West African villages (Turner *et al.,* 2005).

p53 Mutation

One study in Taiwan found that, in the presence of aflatoxin, the *p53* 249ser mutation was significantly more frequent among HBV positive than negative subjects (Montesano *et al.,* 1997) another study in Qidong shows the *p53* 249 mutation is frequent in cirrhosis patients that control patients (Xing-Hua *et al.,* 2003). In the present study 238 samples were analysed for *p53* mutation. Among this 130 samples were Hepatitis B positive and 108 samples were control. We found 29 samples were positive for aflatoxin-albumin adduct and hepatitis B. Among these, 29 aflatoxin-albumin positive samples, 8 samples

are both Hepatitis B positive and *p53* mutation positive (p=0.07). At 10% level of significance, if probability <0.1, then, presence of aflatoxin-albumin adduct and *p53* mutation are dependent, otherwise, independent. Here, we found that presence of afaltoxin and *p53* mutation is dependent in Hepatitis B positive samples. Further epidemiological studies of the interrelationship among aflatoxin exposure, HBV status, and the presence of the p53 249ser mutation could help clarify whether HBV acts as a confounder or as a synergistic partner with aflatoxin.

6. SUMMARY AND CONCLUSION

7. SUMMARY AND CONCLUSION

Aflatoxins are the fungal secondary metabolites produced by *Aspergillus flavus* and related species. Aflatoxins are difuranocoumarin compounds, which include aflatoxin B1, B2, G1, G2, M1, M2 and other derivatives. The International Agency for Research on Cancer (IARC) recognizes aflatoxin B1 (AFB1) as a liver carcinogen. Aflatoxin exposure in humans and animals results from the consumption of contaminated food. Studies on humans implicated AFB1 in several illnesses, most notably liver cancer and growth retardation in children. Synergism was demonstrated between Hepatitis B and C virus infections and hepatocellular carcinoma when fed with aflatoxin contaminated food.

Significant advances have recently been made in the development of methods to measure individual levels of exposure to aflatoxin. AFB1 covalently binds to serum albumin, especially to the amino acid lysine. AFB1-lysine has been identified as a useful quantifiable biomarker in human serum to determine the exposure to aflatoxin B1. In experimental animals, the levels of AFB1-albumin adduct reflects the degree of DNA damage to the liver, the target organ for cancer. The measurement of aflatoxin in humans was shown to provide vital information on the role of AFB1 in the etiology of liver cancer in human and all other diseases including Hepatitis B, Hepatitis C and cirrhosis of liver. This information can further be used to assess the aflatoxin exposure risk among various sections of human populations to implement appropriate intervention measures to minimize the risk of aflatoxin exposure related illnesses. Various serological-based assays [enzyme linked-immunosorbent assays (ELISA) using antibodies] have been developed to determine the levels of aflatoxin adducts in humans. These were based on measuring AFB1-lys adduct in serum.

The IC-ELISA developed in this study could be a potential tool for epidemiological studies to identify vulnerable groups. This test is simple to perform, cost-effective and enables high-throughput analysis.

This study also involved the development and standardization of a method for detection of aflatoxin in human serum. Thus, monoclonal and polyclonal antibodies were produced. It was observed that polyclonal antibodies had high detection limit. These were used to evolve an indirect competitive ELISA method. This is a simple assay that can be applied in field studies to a relatively large number of samples. The protocol to prepare the AFB1-lys and AFB1-oval adduct was standardized *in vitro* by forming AFB1-8,9 epoxide, and the epoxide was conjugated with lysine or ovalbumin. These were used at various concentrations to standardize the ELISA procedure using antibodies produced indigenously. Method for the extraction of albumin from serum fraction was standardized. After albumin hydrolysis with proteinase K, the AFB1-lysine adduct concentration was estimated by ELISA. The detectable limit of AFB1-lys in blood using polyclonal antibody through ELISA was found to be 7.8 pg per 100 µl. The assay has 96±11% recovery of AFB1-lysine adduct in artificially spiked samples. This assay was validated by testing 673 hepatitis B virus positive and cirrhosis of liver patient samples and healthy control samples for the AFB1 exposure. Eighty six of them tested positive for aflatoxin-lysine adduct. Out of this seventeen samples were from healthy control and sixty nine samples were from hepatitis B and cirrhosis of liver positive samples.

The Restriction fragment length polymorphism (RFLP) method was standardized to detect aflatoxin induced *p53* mutation. Two hundred and thirty eight hepatitis B positive patient samples and healthy control samples were analyzed for the presence of *p53* mutation. of these 10 samples were found to have mutation. There was synergistic effect between aflatoxin presence and *p53* mutation in Hepatitis B positive samples. The method standardized in this study would be useful for further studies on screening large number of samples from various sections of populations in India to assess the level of aflatoxin exposure.

In conclusion,

- This study has simplified the preparation of AFB1-ova and AFB1-lys standards that are necessary to use as standards in ELISA assay to determine the aflatoxin concentration in human serum

- Standardized a simple protocol for the preparation of serum samples for aflatoxin analysis
- Produced monoclonal and polyclonal antibodies and standardized IC-ELISA to detect aflatoxin-albumin adduct in human blood
- Validated the antibodies produced during this period for its efficacy and sensitivity, and showed that it is high in titer
- Evaluated 380 hepatitis B virus positive samples and detected 70 samples as positive to aflatoxins. Out of 293 control samples 16 samples are positive to aflatoxins.

Further studies should focus on screening more number of hepatitis B virus positive samples and random population to assess the likely risk of such patients for liver cancer and to suggest appropriate remedial measures through dietary interventions to minimize the risk of liver cancer. Wider analysis by testing various groups of populations from different socio-economic backgrounds and agro-eco regions is necessary to asses the risk of aflatoxin exposure among Indian populations to develop appropriate intervention strategies.

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8. APPENDIX

8. Appendix

8.1. Buffers and reagents for ELISA (Kumar *et al.*, 2004)

8.1.1. Coating buffer (Carbonate buffer, pH 9.6)

Na_2CO_3 - 1.59 g

$NaHCO_3$ - 2.93 g

Distilled water - 1000 ml

8.1.2. Phosphate buffer saline (PBS), pH 7.4

Na2HPO4 2.38 g

KH2 PO4 0.4 g

KCl 0.4 g

NaCl 16.0 g

Distilled water to 2 l

No need to adjust the pH

8.1.3. Phosphate buffer saline Tween (PBS-T)

PBS 1 l

Tween-20 0.5 ml

8.1.4. Substrate buffer (diethanolamine buffer) for ALP system

10% Diethanolamine

Prepare 10% diethanolamine in distilled water and store at 4˚C. Adjust pH to 9.8 with con.HCl. Prepare 0.5 mg/ml p-nitro phenyl phosphate (PNPP) in 10% diethanolamine, pH 9.8 (for each 15 mg table 30 ml substrate buffer is required). This solution should be prepared fresh. Don't store left over buffer.

Note: Diethanolamine is toxic and harmful to eyes. Take necessary care to avoid contact with skin. PNPP convert to p-nitrophenol after reacting with APL. Plates after adding substrate must be handled extremely carefully.

8.2. Buffer for the preparation of aflatoxin adducts and for extraction of albumin

8.2.1. 0.01 M phosphate buffer (pH 7.2)

K_2HPO_4

KH_2PO_4

8.2.2. Preparation of saturated ammonium sulphate (4.1 M at 25 ^{o}C).

Add 761 g of ammonium sulphate to 1L of distilled water and adjust the pH to 7.0.

8.3. Reagents and buffers for Polyacrylamide Gel Electrophoresis (PAGE)

8.3.1. Stack gel buffer (1 M Tris-HCl, pH 6.8)

Tris base 12.1 g

Dissolve in 70 ml distilled water, adjust pH to 6.8 with 1 N HCl and make up to 100 ml with distilled water.

8.3.2. Resolving gel buffer (1 M Tris-HCl, pH 8.8)

Tris base 12.1 g

Dissolve in 70 ml distilled water, adjust pH to 8.8 with 1 N HCl and make up to 100 ml with distilled water.

8.3.3. *Acrylamide/Bis (30:0.8 w/w) mixture

Acrylamide 30 g

Bis acrylamide 0.8 g

Distilled water to 100 ml

Store this solution at 4 0C in amber coloured bottle or wrap the bottle with aluminum foil to avoid exposure to light.

***Precaution:** *Acrylamide is a neurotoxin. Direct contact with skin or inhalation of acrylamide should be avoided. Prepare this solution in fume hood and always wear gloves.*

8.3.4. 10% ammonium persulphate (APS)

APS 100 mg

Distilled water 1 ml

Note: Always prepared fresh solution before use.

8.3.5. TEMED (Sigma, Cat.# T9281)

Store at 4 ^{o}C.

8.3.6. Electrode (running or tank) buffer, pH 8.3

Tris base (25mM) 3 g

Glycine (250 mM) 14.4 g

SDS (1%) 1 g (add this into buffer, for separating proteins)

Distilled water to 1 litre. No need to adjust pH. Store at room temperature.

8.3.7. Plug gel composition (optional)

Note: Plug gel was used to seal the bottom of the gel mould. Use of this depends on the type of electrophoresis unit.

Acrylamide: Bis mixture 1.75 ml

Resolving gel buffer 1 ml

Distilled water 1 ml

TEMED 20 µl

10% APS 40 µl

or 2% agarose prepared in running buffer

8.3.8. Resolving gel composition (12%)

Acrylamide: Bis mixture	12 ml
Resolving gel buffer	11.25 ml
Distilled water	7 ml
10% SDS	300 µl
TEMED	20 µl
10% APS	100 µl

8.3.9. Stacking gel composition (4%)

Acrylamide: Bis mixture	1.5 ml
Stacking gel buffer	1.25 ml
Distilled water	7 ml
10% SDS	100 µl
TEMED	15 µl
10% APS	200 µl

8.3.10. Lamelli buffer

Distilled water	4.57 ml
1 M Tris-HCl buffer pH 6.8	0.63 ml
40% Glycerol	2.5 ml
10% SDS	2 ml
2-mercaptoethanol	0.1 ml
0.5% Bromophenol blue	0.2 ml

Note: Mix acrylamide:bis solution, gel buffer, distilled water and TEMED mix well, then add APS, swirl the flask and immediately pour into the gel mould.

Caution: Unpolymerized acrylamide is a neurotoxin. Gloves should be worn when preparing this solution.

Procedure

1. Assemble the vertical slab gel apparatus in casting mode as per the manufacturer instructions.
2. Pipette 1 ml of the plug gel solution into the gel mould from a corner and allow it to set. **Note:** Some units may not require sealing the bottom with a plug gel.
3. Pour resolving gel solution into the gel mould leaving about 3 cm space for stacking gel. Gently overlay with water. A sharp water-gel interface will be visible with the polymerization of the gel. **Note:** Add 10% SDS as shown in table, if gel is meant for separating proteins.
4. Decant the water overlay by gently tilting the gel mould.
5. Pour stacking gel solution into the gel mould and insert a comb and allow the gel to set.

Care must be taken not to trap air bubbles below the comb's teeth. **Note:** Add 10% SDS as shown in table if gel is meant for separating proteins.

6. Carefully lift the comb straight-up, without disturbing the wells. Wash the wells with water to remove unpolymerised acrylamide.
7. Fill the lower tank of the electrophoresis unit with electrode buffer. **Note:** Add SDS into electrode buffer, if gel is meant for separating proteins.

8. Insert the gel mould into the electrophoresis unit making sure not to trap air bubbles under the gel.
9. Fill the upper tank with electrode buffer. Avoid direct pouring of buffer into the wells.
10. Load the protein samples for separating in the gels.

8.4. Reagents for silver staining of proteins

8.4.1. Fixing solution (Prepared freshly before use)

Glacial acetic acid 3 ml

Methanol 50 ml

distilled H2O 147 ml

8.4.2. DTT wash solution (0.05% Dithiothreitol (DTT)

DTT 5 mg

Distilled H_2O 10 ml

This can be stored as aliquots at -20 ^oC. **Note:** Diluted this to 1:100 freshly before use.

8.4.3. Silver nitrate solution (Prepared freshly before use)

Silver nitrate 400 mg

distilled H_2O 200 ml

8.4.4. Developer solution (Prepare freshly before use)

Na_2CO_3 6 g

Formaldehyde 100 µl

Distilled H_2O 200 ml

8.4.5. Stop solution

Glacial acetic acid 1 ml

Distilled H_2O 99 ml

Staining procedure (all steps performed at room temperature)

1. Gel was placed in a fixing solution for 30 min, with gentle shaking.
2. Gel was rinsed with in distilled H_2O for 3 times, 10 min given for each wash.
3. Then 100 ml of DTT solution was added and kept for shaking for 30 min.

4. Gel was rinsed with distilled H_2O, and placed it in silver nitrate solution for 30 min. Then gel was rinsed quickly with distilled water and placed in developer till bands are clear. Reaction was stopped by adding stopper solution for 20 min.
5. The stopper solution was then replaced it with distilled water.

8.5. Agarose gel electrophoresis

Agarose gels are prepared by melting agarose in the desired buffer until a clear transparent solution is obtained. The molten agarose solution is poured into a mould (boat) and allowed to harden. Upon hardening the agarose forms a matrix, the density of which depends on the concentration of the agarose.

8.5.1. Materials

• Horizontal electrophoresis unit

• Power supply

• Agarose (electrophoresis grade; BioRad, Cat.# 162-0125)

• UV Transilluminator (302 nm wave length)

8.5.2. 10x Electrophoresis buffer (TBE buffer, pH 8.3)

Tris base (0.45 M) 54 g

Boric acid (0.45 M) 27.5 g

0.5 M EDTA, pH 8 (0.01 M) 20 ml

Distilled water to 1 liter

It is not necessary to adjust pH. Sterilize by autoclaving and store at room temperature.

8.5.3. Working solution (0.5x)

To 5 ml of 10x TBE buffer add 95 ml of sterile distilled water. The final concentration of Tris-base, boric acid and EDTA in working solution is 0.045 M, 0.045 M and 0.001 M, respectively.

8.5.4. 5x Sample buffer (Gel loading buffer)

Bromophenol blue (0.25%) 5 mg

Xylene cyanol FF (0.25%) 5 mg

Glycerol (30%) 3 ml

Sterile distilled water to 10 ml

8.5.5. 1% Ethidium bromide solution

Ethidium bromide 100 mg

Distilled water 10 ml

Store in a dark coloured bottle at 4 0C.

8.5.6. Working solution (0.5 µg/ml)**:** To 100 ml water or molten agarose, add 5 µl of 1% ethidium bromide.

Caution: Ethidium bromide is a carcinogen. Gloves should be worn when handling and care must be taken to dispose materials containing this substance.

8.5.7. Procedure

1. Prepare agarose at the desired concentration (w/v) in 1x TBE buffer (for 2% gel, dissolve 2 g agarose in 100 ml buffer) and boil in a microwave oven or on a hot water bath, with intermittent shaking until all the agarose is completely dissolved. Replace evaporation loss with distilled water. **Note:** Ethidium bromide can be directly added into molten agarose [8 µl (0.05µg/ml) /100ml]. This is kind of staining is used for routine analysis. However, if the gel is for estimating molecular size of DNA fragments, do not add ethidium bromide into the gel. It affects DNA migration in gel.
2. Seal the edges of the gel tray with a tape and place the comb at one end of the tray surface.
3. Cool the agarose solution to about 50 0C and pour into the gel tray to a thickness of 4-5 mm and allow the gel to set. **Note:** It will take about 20 min for agarose to harden.
4. Remove the tape and place the gel tray in the electrophoresis unit and fill the unit with 0.5x TBE buffer so that there is 2-3 mm of buffer over the gel surface. Then remove the comb carefully. **Note:** Wells should be towards cathode end (black colour leads). The migration of DNA will be towards anode (red colour leads)
5. Mix 6 µl of loading buffer to 30 µl of PCR product and load slowly into the wells. Avoid overloading of the wells.
6. Load DNA molecular weight marker.
7. Connect electrophoresis unit to the power pack and turn on power supply until the bromophenol blue dye reaches the bottom of the gel. (Approximately 60 min at 100 V, for DNA to migrate 7 cm from the wells in a 1% gel)
8. Remove the gel from the tray and stain in ethidium bromide solution (0.5 µg/ml) in water for 15 min with gentle agitation. Then destain by soaking the gel in water for 5 min.
9. Observe the gel under UV Transilluminator using UV protective goggles or a full safety mask that efficiently blocks UV light. Photograph the gel using an orange filter fitted camera. **Caution:** UV radiation is very dangerous to the skin and particularly to the eyes. **It is absolutely essential to use UV-protective goggles.**

8.6. Definitions of some terms

(Coleman *et al.,* 1992)

Term	Definition
Phagocytic activity	Ingestion of cells or particles (antigen) by phagocytes or the process by which phagocytes engulf the other cells.
Lymphocyte	Mononuclear cell, approximately 7-12 μm in diameter, containing a large, round nucleus with densely packed chromatin and a small thin outer layer of cytoplasm. Several classes of lymphocytes are identified on the basis of surface antigens that they express and their roles in immune responses.
CD4	Diagnostic surface antigen of T_H and T_D cells. This antigen is associated with the T cell antigen recognition receptor and contributes to class II MHC restriction of these cells.
CD8	Diagnostic surface antigen of Tc and Ts cells. This antigen is associated with the T cell antigen recognition receptor and contributes to class I MHC restriction of these cells.
Mutagenic	The agent which can cause mutation
Immunosuppressor	The agent which can suppress the immune system
Hepatocarcinogen	The agent which is responsible for liver cancer
Hepatotoxin	Substances toxic to liver
Degranulation	Process whereby granulocytes discharge the contents of their cytoplasmic granules (lysosomes or specific granules). This occurs during the formation of phagolysosomes by neutrophils or in the discharge of specific granule content of basophils and mast cells.

ANNEXURE-I

THE STUDY ON SERUM AFLATOXIN LEVELS IN LIVER DISORDERS ASSOCIATED WITH INCREASED RISK OF HEPATOCELLULAR CARCINOMA

Informed Consent for participating in the study by providing blood sample for estimation of serum aflatoxin levels.

Date …….

I ……..S/o, D/o ……hereby agree to participate on the above mentioned study and agree to provide blood sample for estimating serum Aflatoxin levels. I have been explained in detail the nature of the study –

1) The aim of the study is to estimate aflatoxin levels in different liver diseases and compare with those who do not have liver disease.
2) The outcome of the study may prove beneficial in taking some preventing measures.
3) I have been assured of confidentiality.
4) I have been given opportunity at my free will to ask questions and seek clarifications.

Signature of the Investigator
Or
Person authorized by Investigator

Signature of the Patient/Representative
authorized by the patient.

Signature of the independent witness

CASE RECORD FORM

Name **Serial No.**

Age **Sex** **Address**

PR No. **IP No.**

DOB

D/o Admission

D/o Discharge

Final Diagnosis **Tel:** **(Landline) :**

Mobile :

Co-Morbid Conditions

Diabetes Type Duration Medication: OHA ☐ Insulin ☐

☐ ☐ ☐ Both ☐ Diet Control ☐

Hypertension Duration Medication _____

☐ ☐

CAD No Yes ☐

COPD/Asthma No Yes ☐

Lifestyle

Physical Activity Sedentary ☐ Moderate activity ☐ Heavy activity ☐

Smoking No ☐ Yes ☐ Packs/day ☐

Alcohol Whiskey ☐ Wine ☐ Beer ☐ Local ☐

Quantity _____ml No. of years

PROTOCOL ON SERUM AFLATOXIN LEVELS IN LIVER DISORDERS ASSOCIATED WITH INCREASED RISK OF HEPATOCELLULAR CARCINOMA

Name			**Serial No.**
Age	**Sex**		**Address**
PR No.	**IP No.**		
DOB			
D/o Admission			
D/o Discharge			
Final Diagnosis		**Tel:**	**(Landline)**
			Mobile

Food Frequency Questionnaire

1. Do you consume the following foods?

a.	Peanuts	Yes	No
b.	Corn	Yes	No
c.	Wheat	Yes	No
d.	Rice	Yes	No
e.	Barley	Yes	No
f.	Soyabeans	Yes	No
g.	Raw milk (unpasturized)	Yes	No
h.	Red Chillies	Yes	No

2. How frequently do you consume these foods?

a.	Peanuts Daily	Alternate days		Weekly once	Rare
b.	Corn	Daily	Alternate days	Weekly once	Rare
c.	Wheat	Daily	Alternate days	Weekly once	Rare
d.	Rice	Daily	Alternate days	Weekly once	Rare
e.	Barley	Daily	Alternate days	Weekly once	Rare
f.	Soyabeans	Daily	Alternate days	Weekly once	Rare
g.	Raw milk	Daily	Alternate days	Weekly once	Rare
h.	Red Chillies	Daily	Alternate days	Weekly once	Rare

3. Cooking practices

a. In what cooked form do you consume peanuts?

Boiled Roasted Chutneys Peanut butter Any other_____

b. In what cooked form do you consume corn?

Boiled Roasted Corn flakes Any other_____

c. In what cooked form do you consume wheat and its products?

Boiled Roasted Baked Fried Any other_____

d. In what cooked form do you consume rice?

Boiled Roasted Fried Any other_____

e. In what cooked form do you consume barley?

Boiled Roasted Barley water Malted beverages Any other___

f. In what cooked form do you consume soyabeans?

Boiled Roasted Baked Any other_____

4. Quantity of foods consumed

	Consumption Gm/kg per month	**No. of person in family**	
Peanuts			
Corn			
Wheat			
Rice			
Barley			
Soyabeans			
Milk (1 cup = 200ml)			
Red Chillies			

5. Source Supermarket Kirana shop Self produced & stored

Govt. fair price shop Others (specify) _____

6. Socioeconomic profile

Employment	Government	Non Govt.	Self
Salary (per month)			
Education	illiterate	Metric	Graduate
House	Own	Rental	Company
Daily transport	4 Wheeler	2 Wheeler	Public transport
Visits – Abroad	None	1-2	>4
Admitted in ward –			
Free general ward	Paying general ward		Non AC
AC			

Kal

Aetiology Profile

Hepatitis B:	Hepatitis C:	Alcoholism:
Autoimmune:	Wilson:	Cryptogenic:
NAFLD related:	Other:	Hepatocellular Ca:

Symptom Profile

Symptom	Date	Symptom	Date
First Symptom Elaborate		Distension of Abdomen	
Hematemesis/ Malena		Febrile illness	
Edema of feet		Encephalopathy	
Jaundice			
Others			

Signs Profile

Jaundice		Ascites	
Pruritic marks		Edema feet	
Hepatomegaly		Lymph nodes	
Splenomegaly		Mass	
Others			

Metabolic Syndrome Profile

Fasting glucose:		Height:	Hip Circum:
Triglycerides:		Weight:	Wt / H :
Total cholesterol:		BMI :	
Blood pressure:		Waist Circum:	

MELD **MADDREYS**: {4.6 x [PT(P)-PT(C)] + Total Bil}

Date	S.Bilirubin	PT (P)	PT (C)	P-C	INR	Creatinine

MELD SCORE _____ MELD 10{0.957Lg(Scr)+0.378Lg(Tbil)+1.12Lg(INR)+0.643}

	1 Point	**2 Points**	**3 Points**	**Patients points**
Bilirubin (mg/dl)	<2	2-3	>3	
Albumin (mg/dl)	<3.5	2.8-3.5	<2.8	
INR	<1.7	1.7-2.3	>2.3	
Ascites	None	Slight or controlled medically	Moderate or severe	
Encephalopathy	None	Stage 1-2	Stage 3-4	

Class A = 5-6 points, B = 7-9 points, C= 10-15 points

Other Investigation Profile **CPT score** _____

UGI Endoscopy: Hb: _____

US Abdomen: TLC: _____

CT ABD / MRI: DLC: _____

X-ray chest: Platelets: _____

Sero Molecular Profile

HBsAg: _____ Anti-HCV: _____ HIV: _____

HBeAg: _____ HCV Genotype: _____ Others _____

Anti-HBe: _____ HCV Quant : _____

HBV Genotype: _____ HBV Quant: _____

Biochemistry Profile (ULN)

S.Bilirubin.T: _____ ( ) Total protein: _____

S.Bilirubin.D: _____ ( ) Alb: _____

ALT (SGPT): _____ ( ) Glob: _____

AST (SGOT): _____ ( ) Urea: _____ ( )

Alk.phosph: _____ ( ) Creatinine: _____ ( )

GGT: _____ ( ) Uric Acid: _____ ( )

BIOPSY PROFILE

MASTER CHARTS

Chart 1: Aflatoxin-lysine positive status in Hepatitis B positive and control samples

S.No	Patient ID	Age	Gender	Diagnosis	Aflatoxin B1-lysine status	AFB1 concentration in pg/mg of albumin
1	AFB01	45	M	HBV	Positive	31.5
2	AFB02	43	M	HBV	Negative	0
3	AFB03	47	M	HBV	Negative	0
4	AFB04	56	M	HBV	Positive	129
5	AFB05	52	F	HBV	Negative	0
6	AFB06	61	M	HBV	Negative	0
7	AFB07	45	M	HBV	Positive	111
8	AFB08	47	F	HBV	Negative	0
9	AFB09	49	M	HBV	Negative	0
10	AFB10	58	M	HBV	Positive	676.5
11	AFB11	49	M	HBV	Negative	0
12	AFB12	53	M	HBV	Negative	0
13	AFB13	56	M	HBV	Negative	0
14	AFB14	48	F	HBV	Negative	0
15	AFB15	41	M	HBV	Negative	0
16	AFB16	58	F	HBV	Negative	0
17	AFB17	48	M	HBV	Positive	403.9
18	AFB18	56	F	HBV	Negative	0
19	AFB19	50	M	HBV	Positive	180.8
20	AFB20	40	F	HBV	Negative	0
21	AFB21	49	M	HBV	Negative	0
22	AFB22	61	F	HBV	Negative	0
23	AFB23	41	M	HBV	Positive	249.1
24	AFB24	50	M	HBV	Negative	0
25	AFB25	56	F	HBV	Negative	0
26	AFB26	45	M	HBV	Negative	0
27	AFB27	49	M	HBV	Positive	393.4
28	AFB28	59	F	HBV	Negative	0
29	AFB29	42	M	HBV	Negative	0
30	AFB30	44	F	HBV	Positive	153.1
31	AFB31	57	M	HBV	Negative	0
32	AFB32	51	M	HBV	Negative	0
33	AFB33	44	M	HBV	Negative	0
34	AFB34	49	F	HBV	Negative	0
35	AFB35	52	F	HBV	Negative	0
36	AFB36	41	M	HBV	Negative	0
37	AFB37	50	M	HBV	Positive	208.4
38	AFB38	59	F	HBV	Negative	0
39	AFB39	52	M	HBV	Positive	68.7
40	AFB40	56	M	HBV	Negative	0
41	AFB41	55	M	HBV	Negative	0
42	AFB42	45	M	HBV	Negative	0

S.No	Patient ID	Age	Gender	Diagnosis	Aflatoxin B1-lysine status	AFB1 concentration in pg/mg of albumin
43	AFB43	58	M	HBV	Positive	597.5
44	AFB44	49	M	HBV	Negative	0
45	AFB45	42	M	HBV	Negative	0
46	AFB46	40	F	HBV	Negative	0
47	AFB47	44	M	HBV	Positive	164.6
48	AFB48	50	M	HBV	Negative	0
49	AFB49	39	F	HBV	Negative	0
50	AFB50	57	F	HBV	Negative	0
51	AFB51	41	M	HBV	Negative	0
52	AFB52	45	F	HBV	Positive	318.2
53	AFB53	49	M	HBV	Negative	0
54	AFB54	58	M	HBV	Negative	0
55	AFB55	47	F	HBV	Negative	0
56	AFB56	42	M	HBV	Negative	0
57	AFB57	59	F	HBV	Negative	0
58	AFB58	46	M	HBV	Negative	0
59	AFB59	48	F	HBV	Negative	0
60	AFB60	40	F	HBV	Negative	0
61	AFB61	53	M	HBV	Negative	0
62	AFB62	50	M	HBV	Negative	0
63	AFB63	45	F	HBV	Negative	0
64	AFB64	50	M	HBV	Negative	0
65	AFB65	46	M	HBV	Negative	0
66	AFB66	49	F	HBV	Negative	0
67	AFB67	41	M	HBV	Negative	0
68	AFB68	48	M	HBV	Positive	54.5
69	AFB69	35	F	HBV	Negative	0
70	AFB70	46	M	HBV	Negative	0
71	AFB71	48	M	HBV	Negative	0
72	AFB72	40	M	HBV	Positive	26
73	AFB73	46	F	HBV	Negative	0
74	AFB74	47	M	HBV	Negative	0
75	AFB75	47	M	HBV	Positive	58.5
76	AFB76	46	F	HBV	Negative	0
77	AFB77	45	M	HBV	Negative	0
78	AFB78	56	M	HBV	Negative	0
79	AFB79	52	F	HBV	Negative	0
80	AFB80	59	M	HBV	Positive	27.5
81	AFB81	46	F	HBV	Negative	0
82	AFB82	48	M	HBV	Negative	0
83	AFB83	40	M	HBV	Negative	0
84	AFB84	55	M	HBV	Positive	32.9
85	AFB85	42	F	HBV	Negative	0

S.No	Patient ID	Age	Gender	Diagnosis	Aflatoxin B1-lysine status	AFB1 concentration in pg/mg of albumin
86	AFB86	55	M	HBV	Negative	0
87	AFB87	54	M	HBV	Negative	0
88	AFB88	59	F	HBV	Positive	115.1
89	AFB89	49	M	HBV	Negative	0
90	AFB90	46	M	HBV	Negative	0
91	AFB91	49	F	HBV	Negative	0
92	AFB92	58	M	HBV	Positive	186.2
93	AFB93	45	F	HBV	Negative	0
94	AFB94	57	M	HBV	Negative	0
95	AFB95	53	M	HBV	Negative	0
96	AFB96	48	F	HBV	Negative	0
97	AFB97	40	M	HBV	Positive	30.7
98	AFB98	49	F	HBV	Negative	0
99	AFB99	53	M	HBV	Negative	0
100	AFB100	47	M	HBV	Negative	0
101	AFB101	54	M	HBV	Negative	0
102	AFB102	45	M	HBV	Negative	0
103	AFB103	41	M	HBV	Negative	0
104	AFB104	54	F	HBV	Negative	0
105	AFB105	46	M	HBV	Negative	0
106	AFB106	53	F	HBV	Positive	121.4
107	AFB107	49	M	HBV	Negative	0
108	AFB108	45	F	HBV	Negative	0
109	AFB109	47	M	HBV	Negative	0
110	AFB110	45	F	HBV	Negative	0
111	AFB111	48	M	HBV	Positive	73.3
112	AFB112	51	M	HBV	Negative	0
113	AFB113	49	M	HBV	Negative	0
114	AFB114	56	M	HBV	Negative	0
115	AFB115	53	F	HBV	Negative	0
116	AFB116	46	M	HBV	Negative	0
117	AFB117	52	M	HBV	Negative	0
118	AFB118	43	M	HBV	Positive	83.7
119	AFB119	41	M	HBV	Negative	0
120	AFB120	56	M	HBV	Negative	0
121	AFB121	45	M	HBV	Positive	119.4
122	AFB122	49	M	HBV	Positive	169.7
123	AFB123	39	M	HBV	Negative	0
124	AFB124	47	M	HBV	Negative	0
125	AFB125	58	F	HBV	Negative	0
126	AFB126	53	F	HBV	Positive	129.5
127	AFB127	61	M	HBV	Negative	0
128	AFB128	56	F	HBV	Negative	0

S.No	Patient ID	Age	Gender	Diagnosis	Aflatoxin B1-lysine status	AFB1 concentration in pg/mg of albumin
129	AFB129	59	M	HBV	Negative	0
130	AFB130	44	M	HBV	Positive	114.2
131	AFB131	29	M	HBV	Negative	0
132	AFB132	40	M	HBV	Negative	0
133	AFB133	43	M	HBV	Negative	0
134	AFB134	60	M	HBV	Negative	0
135	AFB135	76	M	HBV	Negative	0
136	AFB136	35	F	HBV	Positive	132
137	AFB137	65	M	HBV	Negative	0
138	AFB138	53	M	HBV	Negative	0
139	AFB139	44	M	HBV	Negative	0
140	AFB140	32	M	HBV	Negative	0
141	AFB141	38	M	HBV	Negative	0
142	AFB142	42	M	HBV	Positive	57.5
143	AFB143	32	M	HBV	Negative	0
144	AFB144	51	M	HBV	Negative	0
145	AFB145	57	M	HBV	Negative	0
146	AFB146	60	M	HBV	Negative	0
147	AFB147	55	M	HBV	Negative	0
148	AFB148	55	M	HBV	Positive	38
149	AFB149	18	M	HBV	Negative	0
150	AFB150	65	M	HBV	Negative	0
151	AFB151	62	M	HBV	Positive	110
152	AFB152	48	F	HBV	Negative	0
153	AFB153	51	M	HBV	Negative	0
154	AFB154	50	M	HBV	Negative	0
155	AFB155	49	F	HBV	Negative	0
156	AFB156	52	M	HBV	Positive	33.75
157	AFB157	68	M	HBV	Negative	0
158	AFB158	54	M	HBV	Negative	
159	AFB159	74	M	HBV	Negative	0
160	AFB160	60	M	HBV	Positive	185.85
161	AFB161	59	M	HBV	Negative	0
162	AFB162	49	F	HBV	Positive	43.1
163	AFB163	58	F	HBV	Negative	0
164	AFB164	52	M	HBV	Negative	0
165	AFB165	48	F	HBV	Negative	0
166	AFB166	55	M	HBV	Negative	0
167	AFB167	53	M	HBV	Negative	0
168	AFB168	45	M	HBV	Negative	0
169	AFB169	43	M	HBV	Negative	0
170	AFB170	47	F	HBV	Negative	0
171	AFB171	56	M	HBV	Negative	0

S.No	Patient ID	Age	Gender	Diagnosis	Aflatoxin B1-lysine status	AFB1 concentration in pg/mg of albumin
172	AFB172	52	M	HBV	Negative	0
173	AFB173	61	M	HBV	Negative	0
174	AFB174	45	M	HBV	Negative	0
175	AFB175	47	M	HBV	Negative	0
176	AFB176	49	M	HBV	Negative	0
177	AFB177	58	M	HBV	Positive	118.4
178	AFB178	49	M	HBV	Negative	0.0
179	AFB179	53	M	HBV	Positive	250.6
180	AFB180	56	M	HBV	Negative	0.0
181	AFB181	48	M	HBV	Positive	116.0
182	AFB182	41	M	HBV	Negative	0.0
183	AFB183	58	F	HBV	Positive	547.0
184	AFB184	48	M	HBV	Negative	0.0
185	AFB185	56	M	HBV	Negative	0.0
186	AFB186	50	M	HBV	Negative	0.0
187	AFB187	40	M	HBV	Positive	466.5
188	AFB188	49	M	HBV	Negative	0.0
189	AFB189	61	M	HBV	Negative	0.0
190	AFB190	41	M	HBV	Negative	0.0
191	AFB191	50	M	HBV	Negative	0.0
192	AFB192	56	M	HBV	Negative	0.0
193	AFB193	45	F	HBV	Negative	0.0
194	AFB194	49	M	HBV	Positive	117.1
195	AFB195	59	M	HBV	Negative	0.0
196	AFB196	42	F	HBV	Negative	0.0
197	AFB197	44	M	HBV	Negative	0.0
198	AFB198	57	M	HBV	Negative	0.0
199	AFB199	51	F	HBV	Negative	0.0
200	AFB200	44	M	HBV	Negative	0.0
201	AFB201	49	F	HBV	Negative	0.0
202	AFB202	52	M	HBV	Negative	0.0
203	AFB203	41	F	HBV	Negative	0.0
204	AFB204	50	M	HBV	Negative	0.0
205	AFB205	59	M	HBV	Negative	0.0
206	AFB206	52	M	HBV	Negative	0.0
207	AFB207	56	M	HBV	Negative	0.0
208	AFB208	55	M	HBV	Negative	0.0
209	AFB209	45	M	HBV	Positive	419.5
210	AFB210	58	M	HBV	Negative	0.0
211	AFB211	49	M	HBV	Negative	0.0
212	AFB212	42	F	HBV	Negative	0.0
213	AFB213	40	F	HBV	Negative	0.0
214	AFB214	44	M	HBV	Negative	0.0

S.No	Patient ID	Age	Gender	Diagnosis	Aflatoxin B1-lysine status	AFB1 concentration in pg/mg of albumin
215	AFB215	50	M	HBV	Negative	0.0
216	AFB216	39	M	HBV	Negative	0.0
217	AFB217	57	F	HBV	Negative	0.0
218	AFB218	41	M	HBV	Negative	0.0
219	AFB219	45	F	HBV	Negative	0.0
220	AFB220	49	M	HBV	Negative	0.0
221	AFB221	58	M	HBV	Negative	0.0
222	AFB222	47	M	HBV	Positive	394.5
223	AFB223	42	M	HBV	Negative	0.0
224	AFB224	59	F	HBV	Negative	0.0
225	AFB225	46	F	HBV	Negative	0.0
226	AFB226	48	F	HBV	Negative	0.0
227	AFB227	40	F	HBV	Negative	0.0
228	AFB228	53	M	HBV	Negative	0.0
229	AFB229	50	F	HBV	Negative	0.0
230	AFB230	45	M	HBV	Negative	0.0
231	AFB231	50	M	HBV	Negative	0.0
232	AFB232	46	M	HBV	Negative	0.0
233	AFB233	49	M	HBV	Positive	257.0
234	AFB234	41	M	HBV	Negative	0.0
235	AFB235	48	F	HBV	Negative	0.0
236	AFB236	35	M	HBV	Negative	0.0
237	AFB237	46	M	HBV	Negative	0.0
238	AFB238	48	M	HBV	Negative	0.0
239	AFB239	40	M	HBV	Negative	0.0
240	AFB240	46	M	HBV	Negative	0.0
241	AFB241	47	M	HBV	Positive	438.0
242	AFB242	47	M	HBV	Negative	0.0
243	AFB243	46	M	HBV	Negative	0.0
244	AFB244	45	M	HBV	Negative	0.0
245	AFB245	56	F	HBV	Negative	0.0
246	AFB246	52	F	HBV	Negative	0.0
247	AFB247	59	M	HBV	Negative	0.0
248	AFB248	46	M	HBV	Negative	0.0
249	AFB249	48	M	HBV	Negative	0.0
250	AFB250	40	M	HBV	Negative	0.0
251	AFB251	55	M	HBV	Positive	27.5
252	AFB252	42	M	HBV	Negative	0.0
253	AFB253	55	F	HBV	Negative	0.0
254	AFB254	54	M	HBV	Negative	0.0
255	AFB255	59	F	HBV	Negative	0.0
256	AFB256	49	M	HBV	Negative	0.0
257	AFB257	46	F	HBV	Negative	0.0

S.No	Patient ID	Age	Gender	Diagnosis	Aflatoxin B1-lysine status	AFB1 concentration in pg/mg of albumin
258	AFB258	49	F	HBV	Negative	0.0
259	AFB259	58	M	HBV	Negative	0.0
260	AFB260	45	M	HBV	Negative	0.0
261	AFB261	57	M	HBV	Negative	0.0
262	AFB262	53	M	HBV	Negative	0.0
263	AFB263	48	M	HBV	Negative	0.0
264	AFB264	40	F	HBV	Negative	0.0
265	AFB265	49	F	HBV	Negative	0.0
266	AFB266	53	M	HBV	Negative	0.0
267	AFB267	47	M	HBV	Negative	0.0
268	AFB268	54	M	HBV	Negative	0.0
269	AFB269	45	F	HBV	Positive	33.0
270	AFB270	41	M	HBV	Negative	0.0
271	AFB271	54	F	HBV	Negative	0.0
272	AFB272	46	F	HBV	Negative	0.0
273	AFB273	53	F	HBV	Negative	0.0
274	AFB274	49	F	HBV	Negative	0.0
275	AFB275	45	M	HBV	Negative	0.0
276	AFB276	47	F	HBV	Negative	0.0
277	AFB277	45	M	HBV	Negative	0.0
278	AFB278	48	M	HBV	Negative	0.0
279	AFB279	51	M	HBV	Negative	0.0
280	AFB280	49	M	HBV	Negative	0.0
281	AFB281	56	M	HBV	Negative	0.0
282	AFB282	53	M	HBV	Positive	330.0
283	AFB283	46	M	HBV	Negative	0.0
284	AFB284	52	M	HBV	Negative	0.0
285	AFB285	43	M	HBV	Negative	0.0
286	AFB286	41	M	HBV	Negative	0.0
287	AFB287	56	M	HBV	Negative	0.0
288	AFB288	45	M	HBV	Negative	0.0
289	AFB289	49	M	HBV	Negative	0.0
290	AFB290	50	F	HBV	Negative	0.0
291	AFB291	52	M	HBV	Positive	121.5
292	AFB292	49	F	HBV	Negative	0.0
293	AFB293	51	F	HBV	Negative	0.0
294	AFB294	47	M	HBV	Negative	0.0
295	AFB295	44	F	HBV	Negative	0.0
296	AFB296	49	M	HBV	Negative	0.0
297	AFB297	50	M	HBV	Negative	0.0
298	AFB298	46	M	HBV	Positive	603.5
299	AFB299	53	F	HBV	Positive	286.0
300	AFB300	47	M	HBV	Negative	0.0

S.No	Patient ID	Age	Gender	Diagnosis	Aflatoxin B1-lysine status	AFB1 concentration in pg/mg of albumin
301	AFB301	55	F	HBV	Negative	0.0
302	AFB302	48	M	HBV	Negative	0.0
303	AFB303	43	F	HBV	Negative	0.0
304	AFB304	52	M	HBV	Negative	0.0
305	AFB305	41	M	HBV	Negative	0.0
306	AFB306	44	F	HBV	Negative	0.0
307	AFB307	58	M	HBV	Negative	0.0
308	AFB308	56	M	HBV	Positive	42.0
309	AFB309	53	M	HBV	Negative	0.0
310	AFB310	52	M	HBV	Positive	229.0
311	AFB311	58	M	HBV	Negative	0.0
312	AFB312	47	M	HBV	Negative	0.0
313	AFB313	49	F	HBV	Negative	0.0
314	AFB314	52	F	HBV	Positive	19.5
315	AFB315	50	M	HBV	Negative	0.0
316	AFB316	49	M	HBV	Negative	0.0
317	AFB317	46	F	HBV	Negative	0.0
318	AFB318	42	M	HBV	Negative	0.0
319	AFB319	51	F	HBV	Negative	0.0
320	AFB320	54	M	HBV	Negative	0.0
321	AFB321	47	F	HBV	Positive	343.0
322	AFB322	56	F	HBV	Negative	0.0
323	AFB323	58	F	HBV	Negative	0.0
324	AFB324	43	F	HBV	Negative	0.0
325	AFB325	46	F	HBV	Negative	0.0
326	AFB326	49	F	HBV	Positive	36.5
327	AFB327	52	F	HBV	Negative	0.0
328	AFB328	44	M	HBV	Positive	450.0
329	AFB329	53	M	HBV	Negative	0.0
330	AFB330	58	M	HBV	Negative	0.0
331	AFB331	49	M	HBV	Negative	0.0
332	AFB332	58	M	HBV	Negative	0.0
333	AFB333	43	M	HBV	Positive	79.5
334	AFB334	49	F	HBV	Negative	0.0
335	AFB335	51	F	HBV	Negative	0.0
336	AFB336	54	M	HBV	Negative	0.0
337	AFB337	44	M	HBV	Positive	25.5
338	AFB338	48	M	HBV	Negative	0.0
339	AFB339	55	M	HBV	Negative	0.0
340	AFB340	47	M	HBV	Negative	0.0
341	AFB341	39	F	HBV	Negative	0.0
342	AFB342	46	M	HBV	Negative	0.0
343	AFB343	47	F	HBV	Negative	0.0

S.No	Patient ID	Age	Gender	Diagnosis	Aflatoxin B1-lysine status	AFB1 concentration in pg/mg of albumin
344	AFB344	52	F	HBV	Negative	0.0
345	AFB345	46	M	HBV	Negative	0.0
346	AFB346	41	F	HBV	Positive	57.5
347	AFB347	43	F	HBV	Positive	29.5
348	AFB348	56	F	HBV	Positive	76.0
349	AFB349	50	F	HBV	Negative	0.0
350	AFB350	44	M	HBV	Negative	0.0
351	AFB351	52	M	HBV	Negative	0.0
352	AFB352	40	M	HBV	Negative	0.0
353	AFB353	42	M	HBV	Negative	0.0
354	AFB354	53	M	HBV	Negative	0.0
355	AFB355	49	F	HBV	Negative	0.0
356	AFB356	43	M	HBV	Positive	438.0
357	AFB357	45	M	HBV	Negative	0.0
358	AFB358	42	M	HBV	Negative	0.0
359	AFB359	52	M	HBV	Negative	0.0
360	AFB360	40	F	HBV	Positive	622.5
361	AFB361	46	F	HBV	Negative	0.0
362	AFB362	43	M	HBV	Negative	0.0
363	AFB363	48	F	HBV	Negative	0.0
364	AFB364	52	M	HBV	Negative	0.0
365	AFB365	41	M	HBV	Positive	283.5
366	AFB366	58	M	HBV	Negative	0.0
367	AFB367	48	M	HBV	Negative	0.0
368	AFB368	46	F	HBV	Negative	0.0
369	AFB369	51	F	HBV	Negative	0.0
370	AFB370	46	M	HBV	Negative	0.0
371	AFB371	49	F	HBV	Negative	0.0
372	AFB372	47	M	HBV	Negative	0.0
373	AFB373	46	M	HBV	Positive	617.5
374	AFB374	36	M	HBV	Negative	0.0
375	AFB375	53	F	HBV	Negative	0.0
376	AFB376	42	F	HBV	Negative	0.0
377	AFB377	57	M	HBV	Negative	0.0
378	AFB378	44	M	HBV	Positive	234.0
379	AFB379	50	M	HBV	Negative	0.0
380	AFB380	56	M	HBV	Negative	0.0
381	AFB381	44	F	Control	Negative	0.0
382	AFB382	49	M	Control	Negative	0.0
383	AFB383	46	F	Control	Negative	0.0
384	AFB384	41	M	Control	Negative	0.0
385	AFB385	43	M	Control	Negative	0.0
386	AFB386	59	F	Control	Negative	0.0

S.No	Patient ID	Age	Gender	Diagnosis	Aflatoxin B1-lysine status	AFB1 concentration in pg/mg of albumin
387	AFB387	45	M	Control	Negative	0.0
388	AFB388	52	M	Control	Negative	0.0
389	AFB389	43	F	Control	Negative	0.0
390	AFB390	49	M	Control	Negative	0.0
391	AFB391	50	F	Control	Negative	0.0
392	AFB392	52	M	Control	Negative	0.0
393	AFB393	49	M	Control	Negative	0.0
394	AFB394	51	M	Control	Negative	0.0
395	AFB395	47	F	Control	Negative	0.0
396	AFB396	44	F	Control	Negative	0.0
397	AFB397	49	M	Control	Negative	0.0
398	AFB398	50	M	Control	Negative	0.0
399	AFB399	46	F	Control	Negative	0.0
400	AFB400	53	M	Control	Negative	0.0
401	AFB401	47	M	Control	Negative	0.0
402	AFB402	55	M	Control	Negative	0.0
403	AFB403	48	M	Control	Negative	0.0
404	AFB404	43	F	Control	Negative	0.0
405	AFB405	52	M	Control	Negative	0.0
406	AFB406	41	F	Control	Negative	0.0
407	AFB407	44	F	Control	Negative	0.0
408	AFB408	58	M	Control	Negative	0.0
409	AFB409	56	M	Control	Negative	0.0
410	AFB410	53	M	Control	Negative	0.0
411	AFB411	52	F	Control	Negative	0.0
412	AFB412	58	M	Control	Negative	0.0
413	AFB413	47	M	Control	Negative	0.0
414	AFB414	49	M	Control	Negative	0.0
415	AFB415	52	M	Control	Negative	0.0
416	AFB416	50	M	Control	Negative	0.0
417	AFB417	49	F	Control	Positive	322.5
418	AFB418	46	F	Control	Negative	0.0
419	AFB419	42	M	Control	Negative	0.0
420	AFB420	51	M	Control	Negative	0.0
421	AFB421	54	M	Control	Negative	0.0
422	AFB422	47	F	Control	Negative	0.0
423	AFB423	56	F	Control	Negative	0.0
424	AFB424	58	M	Control	Positive	41.0
425	AFB425	43	F	Control	Negative	0.0
426	AFB426	46	M	Control	Negative	0.0
427	AFB427	49	F	Control	Negative	0.0
428	AFB428	52	M	Control	Negative	0.0
429	AFB429	44	F	Control	Negative	0.0

S.No	Patient ID	Age	Gender	Diagnosis	Aflatoxin B1-lysine status	AFB1 concentration in pg/mg of albumin
430	AFB430	53	F	Control	Negative	0.0
431	AFB431	58	M	Control	Negative	0.0
432	AFB432	49	M	Control	Negative	0.0
433	AFB433	58	M	Control	Negative	0.0
434	AFB434	43	M	Control	Negative	0.0
435	AFB435	49	F	Control	Negative	0.0
436	AFB436	51	M	Control	Negative	0.0
437	AFB437	54	M	Control	Negative	0.0
438	AFB438	44	M	Control	Negative	0.0
439	AFB439	48	M	Control	Negative	0.0
440	AFB440	55	F	Control	Negative	0.0
441	AFB441	47	F	Control	Negative	0.0
442	AFB442	39	M	Control	Negative	0.0
443	AFB443	46	F	Control	Negative	0.0
444	AFB444	47	M	Control	Positive	52.5
445	AFB445	52	M	Control	Negative	0.0
446	AFB446	46	M	Control	Negative	0.0
447	AFB447	41	M	Control	Negative	0.0
448	AFB448	43	F	Control	Negative	0.0
449	AFB449	56	F	Control	Negative	0.0
450	AFB450	50	M	Control	Negative	0.0
451	AFB451	44	F	Control	Negative	0.0
452	AFB452	52	M	Control	Negative	0.0
453	AFB453	40	M	Control	Negative	0.0
454	AFB454	42	M	Control	Negative	0.0
455	AFB455	53	F	Control	Positive	74.1
456	AFB456	49	F	Control	Negative	0.0
457	AFB457	43	M	Control	Negative	0.0
458	AFB458	45	M	Control	Negative	0.0
459	AFB459	42	M	Control	Negative	0.0
460	AFB460	52	M	Control	Negative	0.0
461	AFB461	40	F	Control	Negative	0.0
462	AFB462	46	M	Control	Negative	0.0
463	AFB463	43	M	Control	Negative	0.0
464	AFB464	48	M	Control	Negative	0.0
465	AFB465	52	M	Control	Negative	0.0
466	AFB466	41	F	Control	Positive	2.8
467	AFB467	58	M	Control	Negative	0.0
468	AFB468	48	M	Control	Negative	0.0
469	AFB469	46	F	Control	Negative	0.0
470	AFB470	51	M	Control	Negative	0.0
471	AFB471	46	M	Control	Negative	0.0
472	AFB472	49	M	Control	Negative	0.0

S.No	Patient ID	Age	Gender	Diagnosis	Aflatoxin B1-lysine status	AFB1 concentration in pg/mg of albumin
473	AFB473	47	M	Control	Negative	0.0
474	AFB474	46	M	Control	Negative	0.0
475	AFB475	36	F	Control	Negative	0.0
476	AFB476	53	M	Control	Negative	0.0
477	AFB477	42	F	Control	Negative	0.0
478	AFB478	57	M	Control	Negative	0.0
479	AFB479	44	F	Control	Negative	0.0
480	AFB480	50	M	Control	Negative	0.0
481	AFB481	56	F	Control	Negative	0.0
482	AFB482	44	M	Control	Negative	0.0
483	AFB483	49	F	Control	Negative	0.0
484	AFB484	46	M	Control	Negative	0.0
485	AFB485	41	M	Control	Negative	0.0
486	AFB486	43	F	Control	Negative	0.0
487	AFB487	59	M	Control	Negative	0.0
488	AFB488	45	M	Control	Positive	593.5
489	AFB489	52	F	Control	Negative	0.0
490	AFB490	43	M	Control	Negative	0.0
491	AFB491	49	F	Control	Negative	0.0
492	AFB492	50	M	Control	Negative	0.0
493	AFB493	52	M	Control	Negative	0.0
494	AFB494	49	M	Control	Negative	0.0
495	AFB495	51	F	Control	Negative	0.0
496	AFB496	47	F	Control	Negative	0.0
497	AFB497	44	M	Control	Positive	41.0
498	AFB498	49	M	Control	Negative	0.0
499	AFB499	50	F	Control	Negative	0.0
500	AFB500	46	M	Control	Negative	0.0
501	AFB501	53	M	Control	Negative	0.0
502	AFB502	47	M	Control	Negative	0.0
503	AFB503	55	M	Control	Negative	0.0
504	AFB504	48	M	Control	Positive	25.0
505	AFB505	43	M	Control	Negative	0.0
506	AFB506	52	M	Control	Negative	0.0
507	AFB507	41	F	Control	Negative	0.0
508	AFB508	44	M	Control	Negative	0.0
509	AFB509	58	M	Control	Negative	0.0
510	AFB510	56	F	Control	Negative	0.0
511	AFB511	53	F	Control	Positive	49.0
512	AFB512	52	M	Control	Negative	0.0
513	AFB513	58	F	Control	Negative	0.0
514	AFB514	47	F	Control	Negative	0.0
515	AFB515	49	M	Control	Negative	0.0

S.No	Patient ID	Age	Gender	Diagnosis	Aflatoxin B1-lysine status	AFB1 concentration in pg/mg of albumin
516	AFB516	52	M	Control	Negative	0.0
517	AFB517	50	M	Control	Negative	0.0
518	AFB518	49	M	Control	Negative	0.0
519	AFB519	46	F	Control	Negative	0.0
520	AFB520	42	M	Control	Negative	0.0
521	AFB521	51	F	Control	Negative	0.0
522	AFB522	54	M	Control	Negative	0.0
523	AFB523	47	F	Control	Negative	0.0
524	AFB524	56	M	Control	Negative	0.0
525	AFB525	58	F	Control	Negative	0.0
526	AFB526	43	M	Control	Negative	0.0
527	AFB527	46	F	Control	Negative	0.0
528	AFB528	49	M	Control	Negative	0.0
529	AFB529	52	F	Control	Negative	0.0
530	AFB530	44	M	Control	Negative	0.0
531	AFB531	53	M	Control	Negative	0.0
532	AFB532	58	F	Control	Negative	0.0
533	AFB533	49	F	Control	Negative	0.0
534	AFB534	58	M	Control	Negative	0.0
535	AFB535	43	M	Control	Negative	0.0
536	AFB536	49	F	Control	Negative	0.0
537	AFB537	51	M	Control	Negative	0.0
538	AFB538	54	F	Control	Negative	0.0
539	AFB539	44	M	Control	Negative	0.0
540	AFB540	48	M	Control	Negative	0.0
541	AFB541	55	M	Control	Negative	0.0
542	AFB542	47	M	Control	Negative	0.0
543	AFB543	39	M	Control	Negative	0.0
544	AFB544	46	M	Control	Negative	0.0
545	AFB545	47	M	Control	Negative	0.0
546	AFB546	52	F	Control	Negative	0.0
547	AFB547	46	F	Control	Negative	0.0
548	AFB548	41	M	Control	Negative	0.0
549	AFB549	43	F	Control	Negative	0.0
550	AFB550	56	M	Control	Negative	0.0
551	AFB551	50	M	Control	Negative	0.0
552	AFB552	44	M	Control	Negative	0.0
553	AFB553	52	M	Control	Negative	0.0
554	AFB554	40	F	Control	Negative	0.0
555	AFB555	42	F	Control	Negative	0.0
556	AFB556	53	M	Control	Negative	0.0
557	AFB557	49	M	Control	Negative	0.0
558	AFB558	43	M	Control	Negative	0.0

S.No	Patient ID	Age	Gender	Diagnosis	Aflatoxin B1-lysine status	AFB1 concentration in pg/mg of albumin
559	AFB559	45	M	Control	Negative	0.0
560	AFB560	42	M	Control	Negative	0.0
561	AFB561	52	M	Control	Negative	0.0
562	AFB562	40	M	Control	Negative	0.0
563	AFB563	46	M	Control	Negative	0.0
564	AFB564	43	M	Control	Negative	0.0
565	AFB565	48	M	Control	Negative	0.0
566	AFB566	52	M	Control	Negative	0
567	AFB567	41	F	Control	Negative	0
568	AFB568	58	M	Control	Negative	0
569	AFB569	48	M	Control	Negative	0
570	AFB570	46	M	Control	Negative	0
571	AFB571	51	F	Control	Negative	0
572	AFB572	46	F	Control	Negative	0
573	AFB573	49	M	Control	Positive	14
574	AFB574	47	F	Control	Negative	0
575	AFB575	46	M	Control	Negative	0
576	AFB576	36	F	Control	Negative	0
577	AFB577	53	M	Control	Negative	0
578	AFB578	42	F	Control	Negative	0
579	AFB579	57	M	Control	Negative	0
580	AFB580	44	M	Control	Negative	0
581	AFB581	50	M	Control	Negative	0
582	AFB582	56	M	Control	Negative	0
583	AFB583	44	F	Control	Negative	0
584	AFB584	49	M	Control	Negative	0
585	AFB585	46	F	Control	Negative	0
586	AFB586	41	M	Control	Negative	0
587	AFB587	43	F	Control	Negative	0
588	AFB588	59	M	Control	Negative	0
589	AFB589	45	M	Control	Negative	0
590	AFB590	52	M	Control	Negative	0
591	AFB591	43	F	Control	Negative	0
592	AFB592	56	M	Control	Negative	0
593	AFB593	53	F	Control	Negative	0
594	AFB594	52	M	Control	Negative	0
595	AFB595	58	M	Control	Negative	0
596	AFB596	47	F	Control	Negative	0
597	AFB597	49	F	Control	Negative	0
598	AFB598	52	M	Control	Negative	0
599	AFB599	50	F	Control	Negative	0
600	AFB600	49	F	Control	Negative	0
601	AFB601	46	M	Control	Negative	0

S.No	Patient ID	Age	Gender	Diagnosis	Aflatoxin B1-lysine status	AFB1 concentration in pg/mg of albumin
602	AFB602	42	F	Control	Negative	0
603	AFB603	51	M	Control	Negative	0
604	AFB604	54	M	Control	Negative	0
605	AFB605	47	F	Control	Negative	0
606	AFB606	56	M	Control	Negative	0
607	AFB607	58	F	Control	Negative	0
608	AFB608	43	F	Control	Negative	0
609	AFB609	46	M	Control	Negative	0
610	AFB610	49	M	Control	Negative	0
611	AFB611	52	F	Control	Negative	0
612	AFB612	44	M	Control	Negative	0
613	AFB613	53	F	Control	Negative	0
614	AFB614	58	M	Control	Positive	9.1
615	AFB615	49	M	Control	Negative	0
616	AFB616	58	M	Control	Negative	0
617	AFB617	43	F	Control	Negative	0
618	AFB618	49	M	Control	Negative	0
619	AFB619	51	F	Control	Negative	0
620	AFB620	54	F	Control	Negative	0
621	AFB621	44	M	Control	Negative	0
622	AFB622	48	M	Control	Negative	0
623	AFB623	55	M	Control	Negative	0
624	AFB624	47	M	Control	Positive	77.2
625	AFB625	39	F	Control	Negative	0
626	AFB626	46	M	Control	Negative	0
627	AFB627	47	F	Control	Negative	0
628	AFB628	52	M	Control	Negative	0
629	AFB629	46	F	Control	Negative	0
630	AFB630	41	M	Control	Negative	0
631	AFB631	43	F	Control	Negative	0
632	AFB632	56	M	Control	Negative	0
633	AFB633	50	F	Control	Negative	0
634	AFB634	44	M	Control	Negative	0
635	AFB635	52	F	Control	Negative	0
636	AFB636	40	M	Control	Negative	0
637	AFB637	42	M	Control	Negative	0
638	AFB638	53	F	Control	Negative	0
639	AFB639	49	F	Control	Negative	0
640	AFB640	43	M	Control	Negative	0
641	AFB641	45	M	Control	Negative	0
642	AFB642	42	F	Control	Negative	0
643	AFB643	52	M	Control	Negative	0
644	AFB644	40	F	Control	Negative	0

S.No	Patient ID	Age	Gender	Diagnosis	Aflatoxin B1-lysine status	AFB1 concentration in pg/mg of albumin
645	AFB645	46	M	Control	Negative	0
646	AFB646	43	M	Control	Positive	34.5
647	AFB647	48	M	Control	Negative	0
648	AFB648	52	M	Control	Negative	0
649	AFB649	41	M	Control	Negative	0
650	AFB650	58	M	Control	Negative	0
651	AFB651	48	M	Control	Negative	0
652	AFB652	46	F	Control	Negative	0
653	AFB653	51	F	Control	Positive	114.6
654	AFB654	46	M	Control	Negative	0
655	AFB655	49	F	Control	Negative	0
656	AFB656	47	M	Control	Negative	0
657	AFB657	46	M	Control	Negative	0
658	AFB658	36	M	Control	Positive	112.5
659	AFB659	53	M	Control	Positive	91.7
660	AFB660	42	F	Control	Negative	0
661	AFB661	57	F	Control	Negative	0
662	AFB662	44	M	Control	Negative	0
663	AFB663	50	F	Control	Negative	0
664	AFB664	56	M	Control	Negative	0
665	AFB665	44	M	Control	Negative	0
666	AFB666	49	M	Control	Positive	90.5
667	AFB667	46	F	Control	Negative	0
668	AFB668	41	F	Control	Negative	0
669	AFB669	43	M	Control	Negative	0
670	AFB670	59	M	Control	Negative	0
671	AFB671	45	M	Control	Negative	0
672	AFB672	52	M	Control	Positive	159.6
673	AFB673	43	F	Control	Negative	0

Chart 2: P53 mutation positive status in hepatitis B positive and control samples

S.No	Age	Gender	Hepatitis B	Aflatoxin B1	P53 Mutation	AFB1 concentration in pg/mg of albumin
1	45	M	Positive	Positive	Negative	31.5
2	43	M	Positive	Negative	Negative	0
3	47	M	Positive	Negative	Negative	0
4	56	M	Positive	Positive	Negative	129
5	52	F	Positive	Negative	Negative	0
6	61	M	Positive	Negative	Negative	0
7	45	M	Positive	Positive	Negative	111
8	47	F	Positive	Negative	Negative	0
9	49	M	Positive	Negative	Negative	0
10	58	M	Positive	Positive	Positive	676.5
11	49	M	Positive	Negative	Negative	0
12	53	M	Positive	Negative	Negative	0
13	56	M	Positive	Negative	Negative	0
14	48	F	Positive	Negative	Negative	0
15	41	M	Positive	Negative	Negative	0
16	58	F	Positive	Negative	Negative	0
17	48	M	Positive	Positive	Negative	403.0
18	56	F	Positive	Negative	Negative	0
19	50	M	Positive	Positive	Negative	180.0
20	40	F	Positive	Negative	Negative	0
21	49	M	Positive	Negative	Negative	0
22	61	F	Positive	Negative	Negative	0
23	41	M	Positive	Positive	Negative	249.1
24	50	M	Positive	Negative	Negative	0
25	56	F	Positive	Negative	Negative	0
26	45	M	Positive	Negative	Negative	0
27	49	M	Positive	Positive	Positive	393.4
28	59	F	Positive	Negative	Negative	0
29	42	M	Positive	Negative	Negative	0
30	44	F	Positive	Positive	Negative	153.1
31	57	M	Positive	Negative	Negative	0
32	51	M	Positive	Negative	Negative	0
33	44	M	Positive	Negative	Negative	0
34	49	F	Positive	Negative	Negative	0
35	52	F	Positive	Negative	Negative	0
36	41	M	Positive	Negative	Negative	0
37	50	M	Positive	Positive	Negative	208.4
38	59	F	Positive	Negative	Negative	0
39	52	M	Positive	Positive	Negative	68.7
40	56	M	Positive	Negative	Negative	0
41	55	M	Positive	Negative	Negative	0
42	45	M	Positive	Negative	Negative	0
43	58	M	Positive	Positive	Positive	597.5

S.No	Age	Gender	Hepatitis B	Aflatoxin B1	P53 Mutation	AFB1 concentration in pg/mg of albumin
44	49	M	Positive	Negative	Negative	0
45	42	M	Positive	Negative	Negative	0
46	40	F	Positive	Negative	Negative	0
47	44	M	Positive	Positive	Negative	164.6
48	50	M	Positive	Negative	Negative	0
49	39	F	Positive	Negative	Negative	0
50	57	F	Positive	Negative	Negative	0
51	41	M	Positive	Negative	Negative	0
52	45	F	Positive	Positive	Positive	318.2
53	49	M	Positive	Negative	Negative	0
54	58	M	Positive	Negative	Positive	0
55	47	F	Positive	Negative	Negative	0
56	42	M	Positive	Negative	Negative	0
57	59	F	Positive	Negative	Negative	0
58	46	M	Positive	Negative	Negative	0
59	48	F	Positive	Negative	Negative	0
60	40	F	Positive	Negative	Negative	0
61	53	M	Positive	Negative	Negative	0
62	50	M	Positive	Negative	Negative	0
63	45	F	Positive	Negative	Negative	0
64	50	M	Positive	Negative	Negative	0
65	46	M	Positive	Negative	Negative	0
66	49	F	Positive	Negative	Negative	0
67	41	M	Positive	Negative	Negative	0
68	48	M	Positive	Positive	Negative	54.5
69	35	F	Positive	Negative	Negative	0
70	46	M	Positive	Negative	Negative	0
71	48	M	Positive	Negative	Negative	0
72	40	M	Positive	Positive	Negative	26
73	46	F	Positive	Negative	Negative	0
74	47	M	Positive	Negative	Negative	0
75	47	M	Positive	Positive	Negative	58.5
76	46	F	Positive	Negative	Negative	0
77	45	M	Positive	Negative	Positive	0
78	56	M	Positive	Negative	Negative	0
79	52	F	Positive	Negative	Negative	0
80	59	M	Positive	Positive	Negative	27.5
81	46	F	Positive	Negative	Negative	0
82	48	M	Positive	Negative	Negative	0
83	40	M	Positive	Negative	Negative	0
84	55	M	Positive	Positive	Negative	32.9
85	42	F	Positive	Negative	Negative	0
86	55	M	Positive	Negative	Negative	0
87	54	M	Positive	Negative	Positive	0

S.No	Age	Gender	Hepatitis B	Aflatoxin B1	P53 Mutation	AFB1 concentration in pg/mg of albumin
88	59	F	Positive	Positive	Negative	115.1
89	49	M	Positive	Negative	Negative	0
90	46	M	Positive	Negative	Negative	0
91	49	F	Positive	Negative	Negative	0
92	58	M	Positive	Positive	Negative	186.2
93	45	F	Positive	Negative	Negative	0
94	57	M	Positive	Negative	Negative	0
95	53	M	Positive	Negative	Negative	0
96	48	F	Positive	Negative	Negative	0
97	40	M	Positive	Positive	Negative	30.7
98	49	F	Positive	Negative	Negative	0
99	53	M	Positive	Negative	Negative	0
100	47	M	Positive	Negative	Negative	0
101	54	M	Positive	Negative	Negative	0
102	45	M	Positive	Negative	Negative	0
103	41	M	Positive	Negative	Negative	0
104	54	F	Positive	Negative	Negative	0
105	46	M	Positive	Negative	Negative	0
106	53	F	Positive	Positive	Negative	121.4
107	49	M	Positive	Negative	Negative	0
108	45	F	Positive	Negative	Negative	0
109	47	M	Positive	Negative	Negative	0
110	45	F	Positive	Negative	Negative	0
111	48	M	Positive	Positive	Negative	73.3
112	51	M	Positive	Negative	Negative	0
113	49	M	Positive	Negative	Negative	0
114	56	M	Positive	Negative	Negative	0
115	53	F	Positive	Negative	Negative	0
116	46	M	Positive	Negative	Negative	0
117	52	M	Positive	Negative	Positive	0
118	43	M	Positive	Positive	Negative	83.7
119	41	M	Positive	Negative	Negative	0
120	56	M	Positive	Negative	Negative	0
121	45	M	Positive	Positive	Negative	119.4
122	49	M	Positive	Positive	Negative	169.7
123	39	M	Positive	Negative	Negative	0
124	47	M	Positive	Negative	Negative	0
125	58	F	Positive	Negative	Negative	0
126	53	F	Positive	Positive	Negative	0
127	61	M	Positive	Negative	Negative	0
128	56	F	Positive	Negative	Negative	0
129	59	M	Positive	Negative	Negative	0
130	44	M	Positive	Positive	Negative	129.5
131	55	M	Negative	Negative	Negative	0

S.No	Age	Gender	Hepatitis B	Aflatoxin B1	P53 Mutation	AFB1 concentration in pg/mg of albumin
132	47	F	Negative	Negative	Negative	0
133	43	M	Negative	Negative	Negative	0
134	56	M	Negative	Negative	Negative	0
135	42	M	Negative	Negative	Negative	0
136	49	F	Negative	Negative	Negative	0
137	45	F	Negative	Negative	Negative	0
138	49	M	Negative	Negative	Negative	0
139	50	F	Negative	Negative	Negative	0
140	52	M	Negative	Negative	Negative	0
141	49	F	Negative	Negative	Negative	0
142	51	M	Negative	Negative	Negative	0
143	47	F	Negative	Negative	Negative	0
144	44	M	Negative	Negative	Negative	0
145	49	M	Negative	Negative	Negative	0
146	50	M	Negative	Negative	Negative	0
147	46	M	Negative	Negative	Negative	0
148	53	F	Negative	Negative	Negative	0
149	47	M	Negative	Negative	Negative	0
150	55	F	Negative	Negative	Negative	0
151	48	M	Negative	Negative	Negative	0
152	43	F	Negative	Negative	Negative	0
153	52	M	Negative	Negative	Negative	0
154	41	M	Negative	Negative	Negative	0
155	44	M	Negative	Negative	Negative	0
156	58	F	Negative	Negative	Negative	0
157	56	M	Negative	Negative	Negative	0
158	53	F	Negative	Negative	Negative	0
159	52	M	Negative	Negative	Negative	0
160	58	M	Negative	Negative	Negative	0
161	47	F	Negative	Negative	Negative	0
162	49	F	Negative	Negative	Negative	0
163	52	M	Negative	Negative	Negative	0
164	50	F	Negative	Negative	Negative	0
165	49	F	Negative	Negative	Negative	0
166	46	M	Negative	Negative	Negative	0
167	42	F	Negative	Negative	Negative	0
168	51	M	Negative	Negative	Negative	0
169	54	M	Negative	Negative	Positive	0
170	47	F	Negative	Negative	Negative	0
171	56	M	Negative	Negative	Negative	0
172	58	F	Negative	Negative	Negative	0
173	43	F	Negative	Negative	Negative	0
174	46	M	Negative	Negative	Negative	0
175	49	M	Negative	Negative	Negative	0

S.No	Age	Gender	Hepatitis B	Aflatoxin B1	P53 Mutation	AFB1 concentration in pg/mg of albumin
176	52	F	Negative	Negative	Negative	0
177	44	M	Negative	Negative	Negative	0
178	53	F	Negative	Negative	Negative	0
179	58	M	Negative	Positive	Positive	9.1
180	49	M	Negative	Negative	Negative	0
181	58	M	Negative	Negative	Negative	0
182	43	F	Negative	Negative	Negative	0
183	49	M	Negative	Negative	Negative	0
184	51	F	Negative	Negative	Negative	0
185	54	F	Negative	Negative	Negative	0
186	44	M	Negative	Negative	Negative	0
187	48	M	Negative	Negative	Negative	0
188	55	M	Negative	Negative	Negative	0
189	47	M	Negative	Positive	Negative	77.2
190	39	F	Negative	Negative	Negative	0
191	46	M	Negative	Negative	Negative	0
192	47	F	Negative	Negative	Negative	0
193	52	M	Negative	Negative	Negative	0
194	46	F	Negative	Negative	Negative	0
195	41	M	Negative	Negative	Negative	0
196	43	F	Negative	Negative	Negative	0
197	56	M	Negative	Negative	Negative	0
198	50	F	Negative	Negative	Negative	0
199	44	M	Negative	Negative	Negative	0
200	52	F	Negative	Negative	Negative	0
201	40	M	Negative	Negative	Negative	0
202	42	M	Negative	Negative	Negative	0
203	53	F	Negative	Negative	Negative	0
204	49	F	Negative	Negative	Negative	0
205	43	M	Negative	Negative	Negative	0
206	45	M	Negative	Negative	Negative	0
207	42	F	Negative	Negative	Negative	0
208	52	M	Negative	Negative	Negative	0
209	40	F	Negative	Negative	Negative	0
210	46	M	Negative	Negative	Negative	0
211	43	M	Negative	Positive	Negative	34.5
212	48	M	Negative	Negative	Negative	0
213	52	M	Negative	Negative	Negative	0
214	41	M	Negative	Negative	Negative	0
215	58	M	Negative	Negative	Negative	0
216	48	M	Negative	Negative	Negative	0
217	46	F	Negative	Negative	Negative	0
218	51	F	Negative	Positive	Negative	114.6
219	46	M	Negative	Negative	Negative	0

S.No	Age	Gender	Hepatitis B	Aflatoxin B1	P53 Mutation	AFB1 concentration in pg/mg of albumin
220	49	F	Negative	Negative	Negative	0
221	47	M	Negative	Negative	Negative	0
222	46	M	Negative	Negative	Negative	0
223	36	M	Negative	Positive	Negative	112.5
224	53	M	Negative	Positive	Negative	91.7
225	42	F	Negative	Negative	Negative	0
226	57	F	Negative	Negative	Negative	0
227	44	M	Negative	Negative	Negative	0
228	50	F	Negative	Negative	Negative	0
229	56	M	Negative	Negative	Negative	0
230	44	M	Negative	Negative	Negative	0
231	49	M	Negative	Positive	Negative	90.5
232	46	F	Negative	Negative	Negative	0
233	41	F	Negative	Negative	Negative	0
234	43	M	Negative	Negative	Negative	0
235	59	M	Negative	Negative	Negative	0
236	45	M	Negative	Negative	Negative	0
237	52	M	Negative	Positive	Negative	159.6
238	43	F	Negative	Negative	Negative	0