ASSESSMENT OF AFLATOXIN M1 AMONG LACTATING MOTHERS AND THEIR INFANTS IN DAMATURU, YOBE STATE NIGERIA

BY

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BY

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DECLARATION

I hereby declare that the work reported in this dissertation titled '**Assessment of Aflatoxin M1mong Lactating Mothers and their Infants in Damaturu Yobe State Nigeria**, has been carried out by me in the Department of Microbiology, Faculty of Life Sciences, Ahmadu Bello University, Zaria, Nigeria, under the careful supervision of Prof. S.E. Yakubu and Prof O. S Olonitola. The information derived from the literature has been duly acknowledged in the text and a list of references provided.

ALEGBE, Sunday David	_____	_____
Name of Student	Signature	Date

CERTIFICATION

This dissertation entitled "**Assessment of Aflatoxin M1 among Lactating Mothers and their Infants in Damaturu Yobe State Nigeria"**by **Sunday David ALEGBE,** (MSc/Sci/43724/2012-2013) meets the regulations governing the award of degree of Master of Science,Ahmadu Bello University Zaria and is approved for its contributions to knowledge and literary presentation.

Prof. S.E. Yakubu Chairman, Supervisory Committee Department of Microbiology ABU Zaria.	_____ Signature	_____ Date
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Prof. S.Z. Abubakar Dean, School of Postgraduate Studies Ahmadu Bello University Zaria.	_____ Signature	_____ Date

DEDICATION

This dissertation is dedicated to Almighty God, my beloved wife Oluwatomi, and lovely children David, Daniel and Dennis.

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ABSTRACT

Aflatoxin M1 is a biomarker of aflatoxin B1 which is detected in breast milk, a possible risk factor for infant early exposure to Aflatoxin. Aflatoxin M1(AFM1) is a measure of Aflatoxin B1 (AFB1) exposure among lactating mothers.Qualitative and quantitative analyses of breast milk samples were carried out by High Performance Liquid Chromatography (HPLC). Also structured questionnaire was used to determined socio demographic factors of lactating mothers and the type of food they consumed after 72hrs. Samples collected at General Sani Abacha Specialist Hospital Damaturu Yobe State Nigeria showed the occurrence of AFM1 among 100 tested lactating mothers breast milk, 82% of the breast milk samples contained Aflatoxin M1. The occurrence of 93% of AFM1 excreted in their urine shows exposure to the toxin for a short time period. The study showed that male infants had higher percentage of 78.1% exposure and saliva secretion of 81.3% due to male may probable tend to consume more volumes of breast milk than the female.Structured questionnaire was used along side with analytical analysis to determine urinary excretion of AFM1 among lactating mothers and it showed that unemployed mothers had 100% exposure compared to the employed mothers (46.1%) with statistically significant difference. Informal education and primary school leavers had highest exposure of 100% AFM1;the educational qualification of lactating mothers is significantly associated with secretion of AFM1 in breast milk, the highest was informal education AFM1 excreted in urine of lactating mothers within 72hrs of food consumption, showed 97.1% of mothers that took milk were exposed, meat: 100% were exposed, corn meal: 93.4% exposed, also, date: 93%, 'Brabisko/Biski': 30.6%, imported

rice :77.7%, native rice: 93.4% occurrence taken($p< 0.05$) . In relation to some selected socio-demographic factors, the highest exposure concentration occurred among the unemployed and age bracket 18-25 and 34-41 with 0.07µg/L. In the case of excretion in urine, 0.05µg/L AFM1 was detected among unemployed study participants with lower concentration among the age of 42 and above having 0.04 µg/L.The 100 tested infants showed that 63% secreted AFM1 in their saliva and 68% excreted AFM1 in their urine. The concentration of AFM1 in infants indicated short time exposure to Aflatoxin. The value of 0.08 µg/L wasthe highest concentration obtained among 5-6 months of age. Urine samples were also observed among 5-6 months to be 0.07 µg/l and infants ≤ 2 months had 0.04 µg/l in their urine samples. Meanwhile, there was a positive correlation between secretions of AFM1 in the breast milk of lactating mothers and the secretions of AFM1 in the saliva of their infants($r=1.475$, $p=0.039$). The concentrations of AFM1 in all the breast milk samples were higher than the acceptable tolerance level of 0.05 µg/L for infant fresh milk as recommended by the Codex Alimentarius.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

The immune system of the human body is constantly battled with invaders; these invaders are both microorganism only but also their various extracellular by-products, i.e. toxins.Mycotoxins are toxic metabolites produced by many fungal strains. Aflatoxins (AFs) are one of the first recognized and vastly researched mycotoxins in the world (Ghiasian and Maghsood, 2012).

Mothers are exposed to many toxins that can reach their infants through breast milk. One of these toxins is aflatoxins, produced by the fungus,*Aspergillus*.*Aspergillus*colonizes grains, especially in tropical regions where there is high temperature and humidity. Aflatoxins are highly toxic, mutagenic, teratogenic, and carcinogenic. One of theseaflatoxins is aflatoxin B1 that is excreted in breast milk as aflatoxin M1 (AFM1) (Ghiasian and Maghsood, 2012).Mycotoxins are toxic metabolites produced by special fungal strains. They are one of the most potent toxic substances produced by the fungi *Aspergillusflavus*and*A. parasiticus*. (Ghiasian and Maghsood, 2012).Adejumo*et al.* (*2013*) reported that the degree of contamination by AFB_1 and AFM_1 of lactating mothers was low in Ogun State. There was a higher occurrence of AFB_1 contamination in Ogun East Senatorial district. 18% of the breast milk had AFM_1 below the detectable limit, while 16% exceeded it. The socioeconomic status of the mothers significantly influenced aflatoxin contamination. There was a correlation between AFM_1, AFB_1 and socioeconomic status of the mothers.

Humans and animals are generally exposed to AFs viadiet. It is proved that AFs are carcinogenic and may cause growth impairment and immune suppressionin numerous animal species (Hall, 1994; IARC, 2002). AFs have been established in human sera and cord blood of women promptly following birth. Therefore, the trans placental transfer of AF by the feto-placental unit has been established (Denning *et al*., 1990). The high AF exposure of West African children and the effects of this exposure on children's growth have been demonstrated by Gong *et al*.(2004). Moreover, the higher level of AFB1 has been correlated with reduced birth weight and jaundice in neonates (Abdulu*et al*., 1998). The Immunity and different aspects of children's health may significantly be influenced bythe exposure to Aflatoxins, by implication a decrease in salivary IgA in Gambian children exposed to aflatoxin (Turner *et al*., 2003).Kamker*et al*. (2008) cited thatAflatoxin-producing members of *Aspergillus* are common and widespread in nature. They can contaminate many foodstuffs at various stages. Poor harvesting practices, improper drying, handling, packaging, storage, and transport conditions contribute to fungal growth and increase the risk of mycotoxin production. Aflatoxins have a high presence in tropical and subtropical regions where humidity and temperature conditions are optimal for toxin productionKamker*et al*. (2008).

Until now, nearly 18 different types of Aflatoxins have been identified; aflatoxin B1 (AFB1) is considered as oneof the most toxic (Bhet *et al*., 2010). Mammals that ingest AFB1-contaminated diets eliminate into milk amounts of the principal 4-hydroxylated metabolite known as ‘milk toxin’ or aflatoxin M1 (AFM1) (Masri*et al.,* 1974*)*. There is a protein called breast cancer resistance protein (BCRP, Abcg2) that mediates the transfer of Aflatoxins into breast milk. This protein reduces systemic exposure to dietary

carcinogens as Aflatoxins by decreasing their net uptake from intestine and by increasing their hepatobiliary, intestinal, and renal elimination. BCRP is also highly expressed in lactating mammary glands in humans and cows mediating aflatoxins excretion in milk (Van Herwaard, 2006).

One of the oldest named genera of fungi is *Aspergillus*received its name from Micheli in 1729. In viewing the microscopic spore-bearing structure, Micheli was reminded of a device used by the Roman Catholic clergy to sprinkle holy water during a part of the liturgy called the *asperges*

(Ainsworth, 1976, Bunett, 2008).

Good number of scientists working on *Aspergillus*, there is a continuing fascination with their biotechnologicalpotential (Bunett, 2008). In addition to producingnumerous useful extracellular enzymes and organicacids, these moulds also produce secondarymetabolites of importance in biotechnology (Bunett, 2008).

Some of these*Aspergillus*species function as plant and/or animal pathogens (Bunett*etal*, 2008). The word‘Aspergillosis’ is the name given to all animal diseases caused by growth of any member of the genus on a living host. Immunosuppression is generally a prerequisite for systemic *Aspergillus*infections in humans. The incidence of systemic aspergillosis, the most serious form, is on the rise and imposes an increasing medical burden upon hospitals and physicians. Better antifungal drugs and diagnostic methods are needed (Bunett, 2008).

*A. flavus*and*A. parasiticus*, strains display a great deal of qualitative and quantitative difference in their toxigenic properties (Bennett *et al,*2008).

It has been estimated that only about the half of *A. flavus*isolates produce aflatoxins(Klich and Pitt, 1988). The total amount of toxin biosynthesized will vary with the strain of the fungus and with the growth conditions. Moisture, temperature, and insect damage are the most important environmental variables associated with aflatoxin contamination of agricultural commodities (CAST, 2003).

1.2 Statement of Problem

Immunotoxicity is a serious issue in maternal and child health. Contamination risk of breast milk with different pollutants including Aflatoxins is high in today's life conditions (Gurbay*et al*., 2010). The immune system is one of the main targets of aflatoxin as reported by Bianco *et al*. (*2012*). If what is termed "safe" for a child is not always safe; a matter of concern because, six months exclusive breast feeding equal to six months exclusive exposure to Aflatoxin M1. Aflatoxin are highly carcinogenic fungus bio-metabolites which can suppress the immune system of the infants at their tender age. Aflatoxin is hapatotoxic and can be one of the risk factors of liver cancer among mothers and children.

1.3 Justification

Breast milk is a major nutrient for infants, feeding of infants with safe milk is essential; the building of infant immunity will depend on the intake of its major nutrient. Bianco *et al*.(2012) reported that Aflatoxinsdo not induce cytotoxity and apoptosis but affect cell cycle. Aflatoxin M1 (AFM1) may exert interactions affecting immunoreactivity. Breastfeeding may be considered as a risk factor for aflatoxin M1 (AFM1) exposure in early infancy (Atasever*et al*.,2014).

Aflatoxin has been demonstrated in vitro to be cytotoxic on human hepatocytes and its acute toxicity in several species similar to aflatoxin B1 and liver cancer has been related to dietry intake of aflatoxins. Aflatoxins exposure exact effect on human and animals exhibits a high level of genotonic activity on DNA(Makun *etal*, 2012and Shundo *etal*,2006). Also, Aflatoxin B1 contamination at high concentration has been correlated with reduced birth weight and jaundice in infants (Abulu *et al*, 1998). However, the capacities of biotransformation of carcinogens in neonates are generally slower than that of adults (Sadeghi *et al*,2009).

1.4 Aim and Objectives

1.4.1 Aim

The aim this work is to assess the levels of aflatoxinM1 a biomarker for aflatoxin B1consumption among lactating mothers and their infants' in Damaturu, Yobe State, Nigeria.

1.4.2 Objectives

The objectivesare to determine the:

i. Occurrence andconcentration of Aflatoxin M1 in lactating Mothers' breast milk and urine.
ii. Occurrence andconcentration of AflatoxinM1 in infant's saliva and urine.
iii. Some selected socio-demographic characteristic for the mothers and correlate with Aflatoxin associated with Aflatoxin M1 exposure.

iv. Types of food lactating mothers often consumewhich may likely predispose them to Aflatoxin exposure.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 History of Aflatoxin

Aflatoxins discovery began immediately after an outbreak of a disease of turkeys of unknown etiology in England in 1960. The disease was called Turkey "X" disease and was eventually attributed to a toxic groundnut meal imported from Brazil (Blout, 1961 and Bunett*etal*, 2003). From that point, an extensive effort to find the cause eventually elucidated that a species of mold, called *Aspergillus flavus*, was involved and the hepatotoxic products of this mold, found also as components in the toxic groundnut meal, were called aflatoxins (Richard 2008). Aflatoxin were found to be carcinogenic which caused concerns that led to worldwide efforts to determine the relationships of these carcinogens to human disease and determine their occurrence in human foods as well as in animal feeds . The findings showed that the aflatoxins were immunosuppressive resulted in establishing that they were probably underlying causes to other diseases, mostly infectious in nature (Richard 2008).

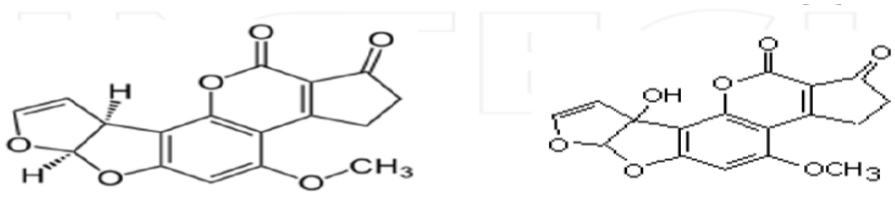
Subsequent efforts led to the finding that aflatoxins can occur pre-harvest and therefore the aflatoxins were no longer only a storage problem. Major crops such as corn, peanuts, cottonseed, and certain tree nuts were frequently found to be contaminated. These findings were cause for the aflatoxin problem to become a large multidisciplinary scientific investigation into various aspects of concern such as eradication, control, analysis, epidemiology, and plant pathology as well as major efforts to determine the nature of animal and human disease caused by the aflatoxins. Present-day investigations

with aflatoxins continue with elimination as a major thrust based on knowledge of the biosynthetic pathway, genetics of host and pathogen, host-parasite-vector interactions, plant breeding, bio control, and selected agronomic practices (Richard 2008).

2.2. Biotransformation

The metabolism of aflatoxin B_1 and the extent to which it binds to cell macro-molecules were compared in liver slices from humans and rats, as rats are more sensitive to the carcinogenicity of aflatoxin B_1. Liver slices were prepared from three human liver samples and incubated with [H^3] aflatoxin B_1at 0.5 µmol/L for 2 h. The rates of formation of oxidative metabolites and of specific binding to cell macromolecules showed significant interindividual variation. The rates of oxidative metabolism of aflatoxin B_1 to aflatoxin Q_1, aflatoxin P_1, and aflatoxin M_1 in the human liver samples were similar to those previously observed in rat liver slices. No aflatoxin B_1–glutathione conjugate formation was detected in the human liver samples, and there was much less specific binding of aflatoxin B_1 to cell macromolecules in the human than in the rat liver slices. For example, the level of binding between aflatoxin B_1 and DNA ranged from 3 to 26% of that in control rats. These results suggest that humans do not form as much aflatoxin $B_1$8,9-epoxide as rats, but they also suggest that humans do not have glutathione-*S*-transferase (GST) isozymes with high specific activity towards this epoxide. Significant individual differences in aflatoxin B_1 metabolism and binding suggest the presence of genetic and/or environmental factors that may result in large differences in susceptibility (Neal *etal*., 1998).

Aflatoxin M_1(fig 2.1b) is usually considered to be a detoxication by-product of aflatoxin B_1(fig 2.1a); it is also the metabolite present in the milk of nursing women who eat foods containing the toxin. Aflatoxin B_1 epoxide has been shown to exist as two stereoisomers—endo- and exo-epoxides—the latter being the DNA-reactive form, and a similar situation may apply to aflatoxin M_1 epoxide. In a study of the metabolism of aflatoxins M_1 and B_1 in vitro in human liver microsomes, there were very limited capacities to catalyseepoxidation of aflatoxin M_1. The small amount of aflatoxin M_1dihydrodiol formed from the epoxide also appeared to have a lower capacity to induce microsomal protein than did aflatoxin B_1dihydrodiol. GST catalysed conjugation of the epoxides of both aflatoxins with glutathione; GST activity was present in mouse cytosol but not in the human liver fraction. The authors concluded that the difference between the genotoxic potency of the two toxins in vivo correlates with their mutagenicity in vitro, metabolic activation and DNA binding (Neal *etal*., 1998). In rats, however, activation of aflatoxin M_1 to the epoxide does not appear to be essential for its acute toxicity. Experiments in human cell lines indicated that cytochrome P450 (CYP) enzymes are involved in the cytotoxicity of aflatoxin B_1 but not of aflatoxin M_1. Studies of the toxicity of aflatoxin M_1 in human lymphoblastoid cell lines expressing or not expressing human CYP enzymes showed a direct effect in the absence of metabolic activation, in contrast to aflatoxin B_1. Aflatoxin M_1 is therefore not strictly a detoxication product of aflatoxin B_1 in biological responses in which cytotoxicity plays a significant role, such as immunotoxicity (Heinonen*etal*., 1996).



a. Aflatoxin B_1 b. Aflatoxin M_1

Fig 2.1 Molecular Structures of Aflatoxin B_1 and M_1

In studies of species sensitivity to the carcinogenicity of aflatoxin B_1, mice were resistant because they constitutively express an *alpha*-GST, which is strongly active against aflatoxin B_1 8,9-epoxide, whereas rats, which do not express such a GST, were sensitive. Human hepatic *alpha*-class GSTs have little capacity to detoxify aflatoxin B_1 8,9-epoxide. The nonhuman primate showed significant constitutive hepatic GST activity towards aflatoxin B_1 8,9-epoxide. GSTs were purified from liver tissue from this species and characterized, and GST cDNAs were cloned by reverse transcriptase-coupled polymerase chain reaction (PCR). A protein, GSHA-GST, was purified by glutathione agarose affinity chromatography, which had stronger aflatoxin B_1 8,9-epoxide-conjugating activity than other GST-containing peaks. The GSHA-GST was shown to belong to the μ class. The authors then showed that two distinct μ-class GST cDNAs have 97% and 98% homology with the human μ-class GSTs hGSTM4 and hGSTM2, respectively. μ-Class GSTs appear to be responsible for most of the conjugating activity of aflatoxin B_1 8,9-epoxide in the liver of *M. fascicularis*. None of the known human μ-class GSTs acts preferentially on the ultimate genotoxicaflatoxin B_1 metabolite exo-aflatoxin B_1 8,9-epoxide, but large interindividual differences in the expression of GST isoforms have been shown in various tissues, and few human livers have been evaluated. The authors concluded that identification of a potential human homologue of GSHA-GST would be relevant to the design of chemointervention strategies to reduce aflatoxin B_1-induced liver cancer in highly exposed populations. Nevertheless, induction of known human GSTs with little or no activity towards the epoxide of aflatoxin B_1 might be ineffective in reducing the genotoxicity of aflatoxin B_1 (Wang *etal*., 2000).

The extreme sensitivity of turkeys to aflatoxin B_1 was studied by measuring microsomal activation of aflatoxin B_1 to the 8,9-epoxide, the putative toxic intermediate, cytosolic GST-mediated detoxication of aflatoxin B_1 8,9-epoxide, and hepatic phase I and phase II enzyme activities in 3-week-old male Oorlop turkeys. Liver microsomes prepared from these turkeys activated aflatoxin B_1 in vitro with an apparent $K_m$of 110 µmol/L and a V_{max} of 1.25 nmol/mg per min. The involvement of CYP 1A2 and, to a lesser extent, 3A4 in the activation of aflatoxin B_1 was assessed with specific mammalian CYP inhibitors. The possible presence of avian orthologues of these CYPs was indicated by activity towards ethoxyresorufin and nifedipine, as well as by western immunoblotting with antibodies to human CYPs. GST-mediated conjugation of 1-chloro-2,4-dinitrobenzene and 3,4-dichloronitrobenzene was demonstrated in cytosol prepared from the turkey livers, but the rate was much lower than that observed in other species(Henry *etal*,2001). The presence of *alpha*- and µ-class GSTs and another aflatoxin B_1 detoxifying enzyme, aflatoxin B_1 aldehyde reductase, was shown by western immunoblotting. Quinone reductase activity was also present in the cytosol. Furthermore, the cytosol showed no measurable GST-mediated detoxication of microsomally activated aflatoxin B_1. Thus, turkeys are deficient in the most crucial aflatoxin B_1detoxication pathway. The authors concluded that the extreme sensitivity of this species to aflatoxin B_1 is due to a combination of efficient aflatoxin B_1 activation and deficient detoxication by phase II enzymes such as GSTs (Klein *etal*., 2000).

The carcinogenicity of aflatoxin B_1, aflatoxicol (aflatoxin L), aflatoxin M_1, and aflatoxicol M_1 (aflatoxin LM_1) was compared in terms of their binding to target organ DNA in rainbow trout. Tritiated compounds were synthesized, dose–response curves for

DNA binding were established, and liver DNA binding indices were calculated for the four aflatoxins after a 2-week dietary intake by trout fry. The adduct levels increased linearly with dietary concentration, with relative DNA binding indices of 21, 20, 2.4, and 2.2 x 10^3 (pmol/mg of DNA)/(pmol/g of diet) for aflatoxin B_1, aflatoxin L, aflatoxin M_1, and aflatoxin LM_1, respectively(Henry *etal*,2001).

In a similar protocol, over 7200 trout fry with an average initial body weight of 1.2 g were used to establish full carcinogen dose–response curves for each aflatoxin and an estimate of the DNA binding index after a single dose. Since trout are very sensitive, $\leq$ 180 µg of each aflatoxin were required. In this analysis, the relative tumorigenic potencies were 1.0 for aflatoxin B_1, 0.94 for aflatoxin L, 0.086 for aflatoxin M_1, and 0.041 for aflatoxin LM_1. When the data were plotted as logit incidence versus L_n adducts (effective dose received), dose–response relationships were found for all aflatoxin adducts, indicating that they are equally tumorigenic, except for aflatoxin LM_1, which was two to three times less potent. Differences in the tumorigenicity of the four aflatoxins are largely or entirely accounted for by differences in uptake and metabolism leading to DNA adduction, rather than to any inherent difference in tumour initiating potency per DNA adduct (Bailey *etal*., 1998).

Since most people are exposed to carcinogens in food intermittently, the effects of intermittent intake of aflatoxin B_1 on hepatic and testicular glutathione was studied in male Fischer 344 rats fed diets containing aflatoxin B_1 at a concentration of 0, 0.01, 0.04, 0.4, or 1.6 ppm at 4-week intervals up to 20 weeks. The control animals were fed an aflatoxin B_1-free NIH-31 diet. Rats eating diets containing 0.01 ppm aflatoxin B_1 did not

show induction of hepatic or testicular GST activity, but intermittent intake of concentrations of 0.04–1.6 ppm significantly increased GST activity. The increase in enzyme activity was proportional to the dose and the length of intake of aflatoxin B_1 (Sahu*etal*., 2000).

2.3 Metabolism of Aflatoxin B1

Biosynthesis of aflatoxin is a complex process that is controlled by genes maintained in a cluster. The transcriptional regulation is controlled by a protein encoded by aflR Chromosomal position effects, as well as a number of other globally acting regulatory genes may be subject to nutritional and environmental control. However there is no evidence for horizontal transfer of the aflatoxin cluster among aflatoxin and sterigmatocystin-producing organisms is available, and no prokaryotic origin for clustered genes has yet been identified. The biological and evolutionary importance of aflatoxin production to the organism is also poorly understood. The clustering of these genes implies that the cluster plays an important role in fungal growth and survival. Otherwise these genes would be rapidly lost, due to genetic drift events and rearrangements. It is not yet understood how aflatoxins contribute to fungal survival since certain non-aflatoxigenic *A. flavus* species appear to compete favorably with aflatoxigenic species for the same niche. Other questions remain: what is the relationship between primary and secondary metabolism, how does stress affect aflatoxin gene expression, particular during fungal interaction with plants. Screening of an Expressed Sequence-Tag (EST) library using microarrays may provide a way to better understand how and why aflatoxins are produced. The *A. flavus* EST/microarray project is being

actively pursued by this Research Unit in collaboration with The Institute for Genomic Research. Information obtained through this genomics project may help in devising strategy for elimination of aflatoxin contamination of crops, and thereby provide a sustainable, healthy food supply (Jiujiang, 2002). Metabolism of Aflatoxin is illustrated in fig. 2.2.

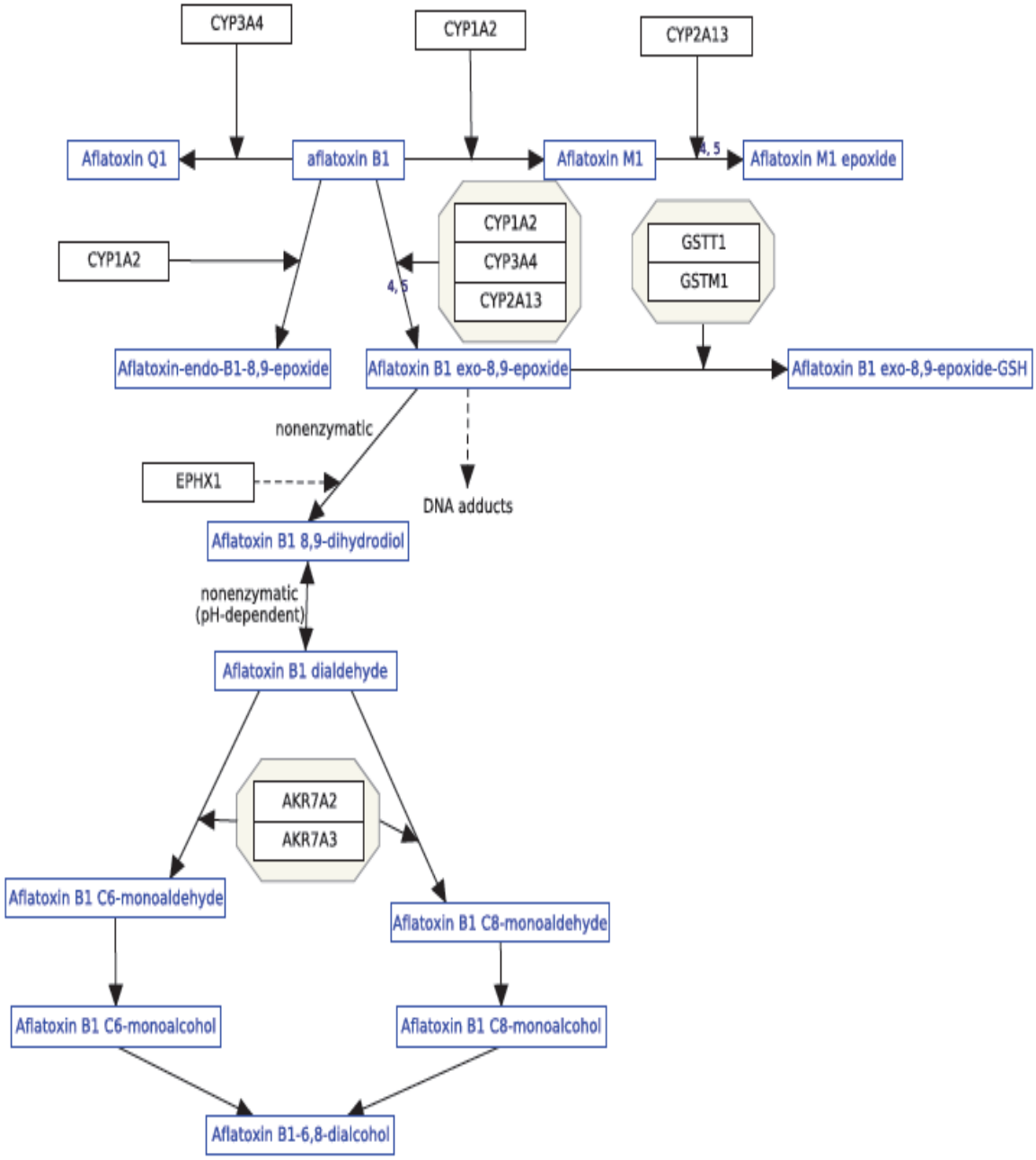


Figure 2.2Pathways for Aflatoxin B1 metabolism in Humans
(www.google.com.ng/search?q=biosynthesis+of+aflatoxin&tbm)

2.4 Degradation of Aflatoxin M_1 in milk

Several possibilities for eliminating or inactivating aflatoxin M_1 in milk, involving chemical and physical treatment, have been investigated. The chemicals that have been studied for their ability to degrade aflatoxin M_1 are limited to those that are permitted as food additives: sulfites, bisulfites, and hydrogen peroxide (Applebaum and Marth, 1982a,b). When raw milk naturally contaminated with aflatoxin M_1 was treated with 0.4% potassium bisulfite at 25 °C for 5 h, the concentration decreased by 45%. A higher concentration of bisulfite was less effective. Aflatoxin M_1 in naturally contaminated milk was not affected by the presence of 1% hydrogen peroxide at 30 °C for 30 min, but addition of hydrogen peroxide at a concentration of 0.05–0.1% with lactoperoxidase reduced the amount by 50 % (Henry*etal.*,2001).

Physical processes that have been explored to remove aflatoxin M_1 from milk include adsorption and radiation. Five per cent bentonite in milk adsorbed 89% of aflatoxin M_1 (Applebaum and Marth, 1982a). In a study of the effects of ultra-violet radiation with and without hydrogen peroxide, the concentration of aflatoxin M_1 was reduced by 3.6–100%, depending on the length of time the milk was exposed to radiation, the volume of treated milk, the presence of hydrogen peroxide, and other aspects of the experimental design (Yousef andMarth, 1985).

The chemical and physical treatments described are not readily applicable in the dairy industry, at least at present, as little is known about the biological safety, or the nutritional value of the treated products. Moreover, the costs of the processes may be considerable and prohibitive for large-scale application. If aflatoxin M_1 cannot be

destroyed or removed readily, it can be excluded from milk only by eliminating aflatoxin B_1 from the diet of animals (Henry*etal.*,2001).

2.5 Immunology of the Oral Cavity

The oral cavity is a unique anatomical structure, characterized by the juxtaposition of soft and hard tissues and which is continuously subject to challenge by the external environment and foreign material. Diseases and disorders caused by oral microorganisms are very common and economically important, in particular dental caries (loss of tooth integrity caused by bacteria-derived organic acids) and periodontitis ('gum disease'; chronic destructive inflammation of the supporting tissues of the teeth caused by anaerobic bacteria). Also, halitosis (oral malodour) is caused by sulphide- producing oral bacteria. Oral diseases secondary to systemic disease, e.g. oral candidiasis,due to acquired immunodeficiency are of increasing clinical importance. As well as a number of autoimmune diseases such as Sjögren's syndrome and bullous pemphigoidhave oral manifestations (Taylor, 2015).

The mouth is well served by numerous major and minor salivary glands and the saliva they secrete is a key component of the host defense against infection in the mouth. Thus, patients with xerostomia ('dry mouth') have more dental plaque and increased risk of periodontitis and candidiasis. The saliva contains many molecular elements which restrict microbial growth: for example lysozyme cleaves bacterial cell walls, lactoferrin complexes iron ions which are an essential microbial nutrient, and histatins inhibit the growth of *Candida albicans* and *Streptococcus mutans* (the latter being an aetiological agent of dental caries). Whereas growth inhibitory elements of the saliva could be

considered as components of innate immunity, salivary IgA is part of the adaptive immune response. This serves to aggregate oral bacteria such as *S. mutans*and prevent the formation of dental plaque, a biofilm on the exposed surface of teeth which comprises a thriving ecosystem for oral pathogens (Taylor, 2015).

The tonsils are immune tissues located towards the back of the mouth; they comprise the palatine-, lingual- and tubal tonsils as well as the adenoids (pharyngeal tonsils) and are collections of lymphoid tissue immediately beneath the epithelium. Together the different tonsillar tissues form a ring of lymphoid tissue known as Waldeyer's ring which serves to protect the opening to the pharynx. The tonsils are often a site of bacterial infection, likely because the clefts in which they appear are a site of collection of debris; this can lead to recurring chronic inflammation and tonsillar enlargement (tonsillitis) (Taylor, 2015).

The anatomy and microanatomy of the tissues which surround and support the teeth (the peridontium) is complex and susceptible to acute and chronic inflammation caused by plaque bacteria which accumulate in the space between the tooth and the gum (the gingival sulcus). Fortunately, the periodontium has a number of host defence elements including the gingival epithelium which prevents bacterial adhesion by constantly shedding keratinocytes into the oral cavity ('cell turnover') and protection against invasion by having a substantial keratin component (unlike other tissues of the oral and gastrointestinal mucosa). The connective tissues of the periodontium are highly vascular, facilitating vascular leukocyte emigration in response to infection and the gingival sulcus is bathed with a serum exudate (gingival crevicular fluid, GCF). The latter carries the

entire key molecular (complement components, antibodies) and cellular (neutrophils and plasma cells) components of the immune response necessary to prevent tissue invasion by the sub-gingival plaque bacteria. The destructive, chronic inflammation that manifests clinically as peridontontis is caused by an excessive and inappropriate immune response to pathogenic plaque bacteria, coupled with a failure of the normal processes that limit inflammation and drive tissue repair (Taylor, 2015).

Interest in oral immunology has heightened in recent years with the recognition that there are clinical associations between periodontitis and systemic diseases with inflammatory components (and in particular diabetes). Also, saliva which mixes with GCF in the oral cavity, is a rich and non-invasive source of disease biomarkers such as cytokines, tissue destructive enzymes and pathogenic microorganisms, as well as DNA for studies of genetic associations and pharmacogenomics (Taylor, 2015).

There are numerous small salivary glands within the tongue, lips cheeks and palate. Humans also have four large glands termed the parotid, submaxillary, submandibular and sublingualsalivary glands. Collectively, the primary function of these glands is the production and secretion of saliva. The components of saliva are produced by serous and mucous acinarcells within the glands, and saliva is then drained though a network of ducts into the oral cavity (Humphreys, 2015).

Aside from the production of saliva, the large salivary glands also help protect us from the numerous microbes that we are constantly exposed to through our mouth. Plasma B cells reside in the salivary glands and produce IgA antibody which is then secreted in the

saliva. IgA binds the mucus layer that covers the epithelia lining of the oral cavity, thus providing a barrier against potentially dangerous pathogens (Humphreys, 2015).

As part of the oral mucosal system, the salivary gland is also exposed to a large number of harmless antigens in the form of foods. To ensure that our white blood cells do not react to these proteins, immune responses in the salivary glands are tightly regulated. One mechanism by which regulation occurs is through the production of a suppressive immune molecule (cytokine) called transforming growth factor–b (TGF–b). TGF–b limits the expansion of T and B cells and inhibits their ability to induce inflammation, therefore preventing accidental tissue damage that may otherwise be triggered by food (Humphreys, 2015).

Mechanisms employed by our immune system to stop reactivity to harmless antigens can also be exploited by unwanted pathogens. Viruses including human cytomegalovirus (HCMV) and Epstein-Barr virus (EBV) can spread via infected saliva. In the case of cytomegalovirus, virus infection of the salivary gland triggers the production of another immune suppressive cytokine called interleukin-10 (IL-10). Like TGF-b, IL-10 can inhibit the accumulation and function of T cells in the salivary glands. Because T cells are required to kill cytomegalovirus, IL-10 mediated inhibition of these cells results in persistent viral replication in, and dissemination from, the salivary glands (Humphreys, 2015).

2.6 Composition and Functions of Saliva:

Saliva is diluted fluid, over 99% water. The concentrations of dissolved solids(organic and inorganic) are characterized by wide variation, both between individuals and within a single individual.

The relative proportion contributed to the whole saliva by each of the gland pairs depends on the degree of stimulation. Thus, under resting conditions, the submandibular glands contribute 69%, the parotid glands 26%, and the sublingual glands 5% (mean values) of the total secretion derived from these three major gland pairs. Under conditions of increased exogenous stimulation, the submandibular glands again account for the largest and the sublingual glands for the smallest fraction of the total secretion from the major glands, but the relative proportion of the total secretion contributed by the parotid glands increases. Thus, the submandibular glands contribute 63.7%, the parotid glands 34%, and the sublingual glands only 2.8%. It has also been established that the mucosal and labial glands in the oral cavity make some contribution to the total volume of saliva under stimulated conditions.(Young and Schneyer, 1981)

2.6.1. Organic composition of saliva proteins

Proteins comprise the bulk of the organic content of saliva but identification of each of them has been more difficult. In studies on human parotid saliva, the presence of at least 20 separate protein fractions has been reported, while in submandibular saliva 21 fractions have been found. (Young and Schneyer, 1981)

Salivary proteins: protective properties

Alpha-Amylase

The major digestive enzyme of saliva,80% is synthesized in the parotid glands, 20% in the sub-mandibular glands. It is the most abundant, accounting for as much as 40-50% of the total salivary gland-produced protein having 60-120mg/100ml in parotid saliva, (30% of parotic saliva) contains 25mg/100ml in submandibular saliva ;Hydrolyzes alpha-1,4 bonds of starches such as amylase and amylopectin .Maltose is the major end-product (20% is glucose). There are several isoenzymes present, both glycosylated and non-glycosylated, with molecular weights ranging from 54 to 57 kDa, depending on the degree of glycosylation (Amerongen*etal*., 2004).

Free amino acids

Free amino acids have found to be in low concentrations. It is less than 0.1mg/100ml (Amerongen*etal*., 2004).

Urea

Urea is also one of saliva organic fluid which is about 12-20mg/100ml, this hydrolysed by many bacteria with the release of ammonia, leading to a rise in pH(Amerongen*etal*., 2004).

Carbohydrates

Normally only traces of free carbohydrates exist in saliva. Glucose present at concentrations 0.5-1mg/100ml, but may be raised in diabetics (Amerongen*etal*., 2004)

Lipids

Lipids are secreted by major salivary glands with concentration 80-100mg/l. and comprised of neutral lipids (about 75%), glycolipids (20-30%) and phospholipids (2-5%).Neutral lipids are mainly free fatty acids, cholesterol, monoglycerides, diglycerides and triglycerides (Amerongen*etal*., 2004). Minor salivary glands contain more lipids (about 400mg/l), glycolipids forming the major group. Majority of lipids in all saliva secretions are associated with proteins (Amerongen*etal*., 2004).

2.7 Inorganic composition of saliva

Hydrogen ions

Hydrogen ions in saliva have several sources of origin: secretion via glands as inorganic or organic acids, produced by oral microbiota, taken into oral cavity in acidic drinks (Fejerskov and Kidd, 2008).The concentration of H+ in saliva has the greatest influence on the chemical reactions in the oral cavity:equilibria between calcium phosphate in dental hard tissue and surrounding liquid phase; solubility, as well as activity, of important salivary enzymes, the variable sources of hydrogen ions as well as its ability to complex with many substances makes the acid-base balance a complex and mutable process for more information refer to asbuffering capacity of saliva (Fejerskov and Kidd, 2008).

Calcium

The concentration of calcium in saliva is influenced by salivary flow rate, calcium is excreted by the glands together with proteins into the lumina of the acini by active transport. Concentration strongly affected by circadian rhythm (Fejerskov and Kidd, 2008,).Eithercirculating as Ca++ ion or as bound calcium (to phosphate, bicarbonate,

small organic ions and macromolecules) depending on salivary pH value.Specialroles of macromolecules such as statherin and histidine in oral calcium homeostasis, normal ratio of Ca++ to bound calcium is 1:1, with Ca++ concentration increasing with decreasing pH; most salivary calcium is in ionised form when pH is less than 4(Fejerskov and Kidd, 2008,).

Inorganic Phosphate

Inorganic phosphate in saliva occur as phosphoric acid H_3PO_4 and its conjugates:H_2PO^{-4},HPO_4^{-2} and $PO_4^{-,3}$. Its concentration is affected by salivary flow rate as well as salivary pH, and also (to a smaller extent compared to calcium) circadian rhythm: concentrations of each type dependent on salivary pH; decreased pH leads to decreased concentration of the tertiary ion; with increased flow rate decreases total inorganic phosphate concentration(Fejerskov and Kidd, 2008,).

Depending on pH, inorganic phosphate can be complexed to inorganic ions or proteins. Less than 10% forms dimer form pyrophosphoricacid - an inhibitor of calcium phosphate precipitation; andinfluences calculus formation (Fejerskov and Kidd, 2008,). Functions of inorganic phosphate include:contributes to solubility product of calcium phosphate, which is crucial in maintaining tooth structure; important as a buffer; an essential nutrient for oral microflora for metabolic pathways(Fejerskov and Kidd, 2008,).

Fluoride

The concentration of fluoride in saliva depends on fluoride in the environment, such as fluoridated drinking water and dental products used for caries prophylaxis. Basal concentration of fluoride is less than 1 micromol per litre, but can be much higher in places where levels of fluoride in drinking water are high. Fluoride also enters saliva via

facilitated transport over membranes of salivary gland tissue. Clearance rate of inorganic fluoride is dependent on salivary flow rate (Fejerskov and Kidd, 2008).

2.7.1. Factors that influence salivary composition

1. Non-Dietary

Source of saliva:

The main electrolytes in human saliva are sodium, potassium, calcium, chloride, bicarbonate, and inorganic phosphate; -There are quantitative differences in the relative proportions of these electrolytes in the major salivary gland secretions. For instance, parotid saliva is relatively low in calcium and high in phosphate as compared with submandibular and sublingual secretions. -Types of proteins and their concentrations are different in the different secretions, eg, most of the salivary amylase is derived from the parotid glands (Dawes 1970, Fejerskov and Kidd, 2008).

Flow rate.

Flow rate has a decided influence on salivary composition. In general, as the flow rate is increased slightly above the unstimulated rate, sodium and bicarbonate concentrations and pH increase, whereas potassium, calcium, phosphate, chloride, urea, and protein concentrations decrease. At higher flow rates, sodium, calcium, chloride, bicarbonate, and protein concentrations and pH increase, whereas the phosphate concentration decreases and the potassium concentration shows little further change. The effect of flow rate on the composition of saliva has been studied in greatest detail with parotid saliva, but limited studies suggest that these findings also apply to the other major salivary gland secretions. Thus, flow rate is a critical variable in any study of the effects of diet on saliva (Dawes 1970, Fejerskov and Kidd, 2008).

Duration of stimulation

When the flow rate of stimulated parotid saliva is maintained constant for several minutes, the composition of the saliva changes considerably with duration of stimulation. Although almost all components of saliva show a change in concentration during the first one or two minutes of stimulation, the concentrations of total protein, calcium, bicarbonate, and chloride and the pH, are particularly affected and may not achieve steady state levels even after 15 minutes of stimulation at constant flow rate (Dawes 1970). Total protein, calcium, and bicarbonate concentrations and pH increase with duration of stimulation, whereas the chloride concentration decreases in proportion to the rise in bicarbonate concentration. -Hence, in any study of the effect of diet on the composition of stimulated saliva, the duration of stimulation must be standardized (Dawes 1970, Fejerskov and Kidd, 2008).

Nature of the stimulus.

Studies have shown that the nature of the stimulus decidedly influenced the protein concentration in parotid saliva in some subjects. Similar results have been reported by Caldwell and Pigman for submandibular saliva.Other studies have shown that the salivary glands respond differently to electric, pharmacologic, and gustatory stimuli (Dawes 1970).

Plasma composition.

Many components of saliva collected under standardized conditions, a positive correlation exists between the concentrations in plasma and saliva for calcium, urea, and potassium, whereas the phosphate concentration in human saliva is relatively independent of the concentration in plasma.At a constant flow rate, the bicarbonate concentration in

human saliva has been found to be directly related to plasma PCO_2 (Dawes 1970, Fejerskov and Kidd, 2008).

Time of day.

The time of day at which saliva samples are collected can have a decided influence on both salivary flow rate and composition. Thus in any study of the effect of diet on the composition of saliva it is always necessary to collect samples in day time (Dawes 1970).

Dietary

Foods that require vigorous mastication or that are highly flavored will cause a decided increase in salivary flow rate. The increase in flow rate alone will affect salivary composition(Dawes 1970, Fejerskov and Kidd, 2008).

Systemic effects of diet: consumption of a particular diet causes either an immediate specific effect on salivary composition that is not attributable to a change in flow rate, or a long term change in salivary flow rate, or composition that may take some time to develop(Dawes 1970).

Flow rate.

The degree of functional stimulation is related to size and activity of the salivary glands. Experiment showed that moderate reductions in food intake caused hypertrophy of the rat parotid gland, which was attributable to increased storage of secretory proteins. It was also found that salivary glands increased in size when the diet of rats was supplemented by non-nutritive cellulose. The feeding of pancreatin to rats is known to cause salivary gland enlargement which may be associated with an increased flow rate (Dawes 1970, Fejerskov and Kidd, 2008).

Fluoride.

The concentration of fluoride in saliva is only about 0.01 ppm. Although it has been shown that oral ingestion of 1 to 10 mg of fluoride can cause an increase in salivary fluoride levels. There appears to be little data on the relation of salivary fluoride concentration to the level of dietary fluoride intake at more normal levels of intake (Dawes 1970, Fejerskov and Kidd, 2008).

Urea.

Plaque microorganisms readily produce ammonia from urea to cause a rise in plaque pH. The pH of plaque from fasting subjects is always higher than that of the adjacent unstimulated saliva, which suggests that in the absence of an external source of carbohydrate, the physiologic levels of urea in saliva are sufficient to allow the plaque microorganisms to maintain a positive pH differential between plaque and saliva. The level of urea in saliva is directly related to the level in blood similarly the level of urea in blood is directly related to the level of protein intake. Thus diets high in protein might be expected to maintain relatively high salivary urea levels. Most of the experiments on the effect of diet on saliva have been confounded because of inadequacies in experimental design such as too few subjects, lack of control subjects, and inadequate standardization of the saliva collection technique (Dawes 1970Fejerskov and Kidd, 2008).

2.8 Salivary glands

Saliva is the mixed glandular secretion which constantly bathes the teeth and the oral mucosa. It is constituted by the secretions of the three paired major salivary glands; the parotid, submandibular and sublingual. It also contains the secretions of the minor salivary glands, of which there are hundreds contained within the submucosa of theoral

mucosa and some gingival crevicular fluid (Whelton, 2008). Below are figures illustrations of the glands (Plate 1):

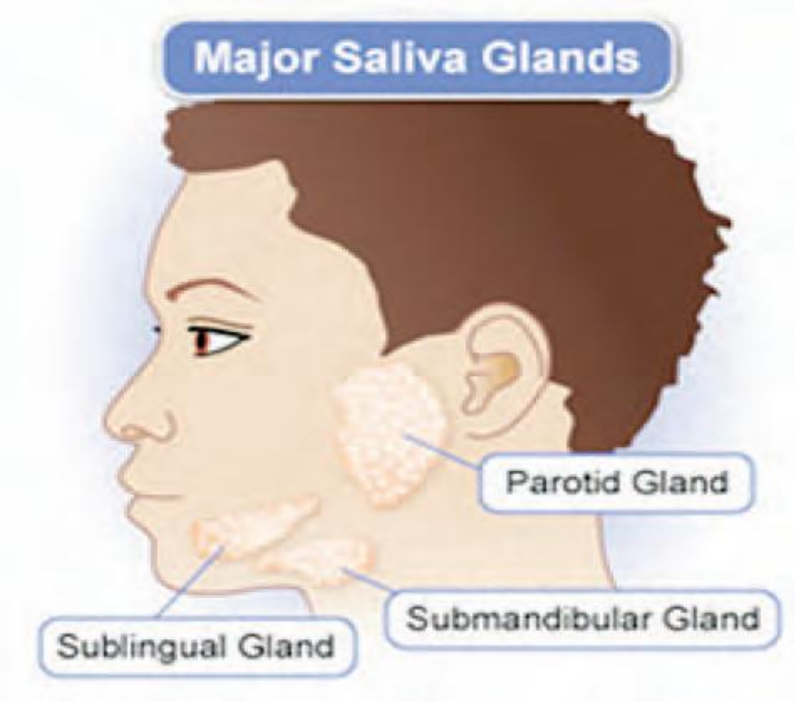


Plate 2.1 Extract from Almeida, *et al.*(2008)

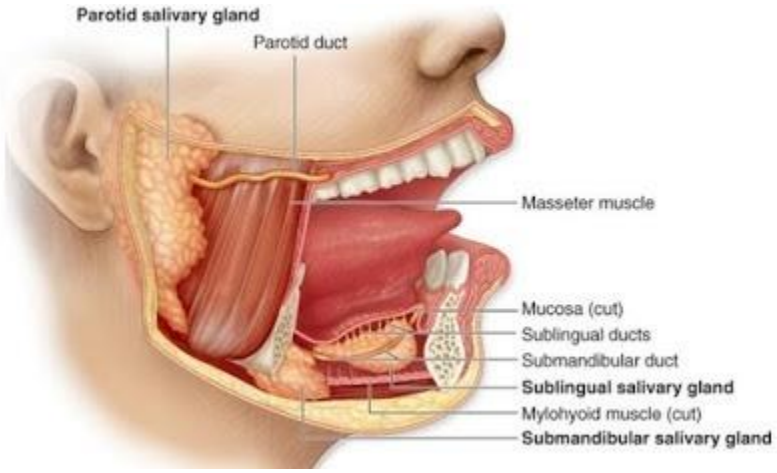


Plate2.2TheHuman Illustration of Major Salivary Gland Almeida, *et al*., (2008).

Plate 2.1 and 2.2 Shows the Major Salivary Glands of the Human Body.

2.9. Analytical methodsof Aflatoxin M1

2.9.1 Screening tests for Aflatoxin M1

Early detection of aflatoxin M_1 and removal of small lots of contaminated milk can prevent contamination of much larger volumes. Screening methods are particularly useful if they can be carried out quickly, easily, and economically. They should allow detection of concentrations of aflatoxin M_1 in milk as low as those detected by the ultimate quantitative methods. Theoretically, screening methods should never give false-negative results.

The screening tests used for aflatoxin M_1 in milk and milk products are usually immunochemical. For aflatoxin M_1, both radioimmunoassays and enzyme immunoassays have been developed (Frémy and Chu, 1989), although enzyme immunoassays are used more often.

The protocol for radioimmunoassay of aflatoxin M_1 usually includes simultaneous incubation of a test solution containing an unknown amount of aflatoxin M_1 (or a standard solution of a known amount of aflatoxin M_1 in phosphate buffer) with a constant amount of labeled aflatoxin and its specific antibody. Free and bound labeled aflatoxins are then separated, and the radiolabel on aflatoxin is determined. The aflatoxin M_1 concentration of the test solution is determined by comparing the results to a standard curve, which is established by plotting the ratio of bound and the initial (total) amount of labeled aflatoxin multiplied by 100 (% binding) versus the concentration of aflatoxin M_1 standard (Chu, 1984). Radioimmunoassay has found little use in routine investigations of

aflatoxin M_1 in milk. An exceptionwas investigatedon milk samples in Thailand (Saitanu, 1997). There have been no collaborative studies on radioimmunoassays for aflatoxin M_1.

Most enzyme immunoassays for aflatoxin M_1 are heterogeneous, with separation of the immunocomplex and the unreacted material. One of the commonest heterogeneous enzyme immunoassays, the ELISA, is generally used to determine aflatoxin M_1. Several direct competitive ELISAs for aflatoxin M_1 are available commercially. The 96-well microtitre plate assay is most commonly used for quantitative measurements. ELISAs for aflatoxin M_1 are usually designed for rapid screening. For legal purposes, positive results in an ELISA require confirmation by an accepted reference method such as HPLC (Henry *etal,* 2001).

One successful use of a direct competitive ELISA, in which the results were confirmed by a validated HPLC method, involved ELISA for the determination of aflatoxin M_1 in pasteurized milk, infant formula, powdered milk, and yoghurt (Kim *etal*., 2000). A valuable addition was comparison of the ELISA with the validated HPLC method of Ferguson-Foos and Warren (1984). Kim *etal*. (2000) concluded that the results for Aflatoxin M_1 obtained with ELISA were similar to those obtained by HPLC. In a study in which ELISA and HPLC with immunoaffinity purification were compared, the latter technique was found to be superior (Biancardi, 1997). An interesting development is a portable field test involving a patented, membrane-based flow-through enzyme immunoassay (Sibanda*etal*., 1999), which can be carried out on farms. The kit comprises a nylon membrane spotted with anti-mouse antibodies, a plastic snap-fit device, absorbent cotton wool, mouse monoclonal antibodies against Aflatoxin M_1, and Aflatoxin

B_1–horse radish peroxidase conjugate. Clean-up is done on an immunoaffinity column(Sibanda*etal*., 1999).

Techniques other than immunochemical procedures can, in principle, be used for rapid analysis of milk and milk products for Aflatoxin M_1. One such technique involves electrochemical flow injection monitoring on filter-supported bilayer lipid membranes (Andreou andNikolelis, 1998). The method has not been formally validated.It has been recommended that extensive collaborative studies be conducted to validate the performance characteristics of immunoassays, including their reproducibility and repeatability, accuracy, sensitivity, limits of detection, specificity, and selectivity (Frémy and Chu, 1989). This recommendation was echoed in 2000 (van Egmond, 2000), as no such studies have been carried out by AOAC International, the organization responsible for collaborative studies of methods of analysis (International Union of Pure and Applied Chemistry, 1989). The International Dairy Federation (1999) has produced a guideline document that outlines the parameters necessary for the evaluation and validation of competitive enzyme immunoassays for quantitative determination of Aflatoxin M_1 in milk and milk products.

2.9.2 Quantitative methods for Aflatoxin M1

Quantitative analytical methods for Aflatoxin M_1 usually follow the general pattern for mycotoxin assays, i.e. extraction, clean-up, concentration, separation, detection, and quantification. A homogeneous sample of Aflatoxin M_1 in milk is easily obtained, because the toxin is distributed evenly in fluid milk. The initial problem encountered in analyzing milk is the extraction step. Because milk is a complex natural product,

Aflatoxin M_1 is not easily extracted and purified for final assay, owing (partly) to adsorption of Aflatoxin M_1 to casein proteins. A process is required to separate Aflatoxin M_1 from milk easily, efficiently, and economically. Once purified extracts are obtained, the concentration of Aflatoxin M_1 can be determined in one of several ways. Most quantitative methods involve thin-layer chromatography (TLC) or HPLC. Aflatoxin M_1 is a weakly polar component and is extractable with solvents such as methanol, acetone, chloroform, or combinations of these solvents with water. In practice, the choice of solvent depends on the clean-up and the separation procedure (Adejumo *et al*, 2013).

The quantitative methods that have been developed and validated for Aflatoxin M_1 in milk and milk products were originally designed to analyze milk powder. Milk was spray-dried or lyophilized to preserve its shelf life and to reduce sample bulk. Various mixtures of methanol and water (Masri *et al*., 1968, 1969a; Fehr et al., 1971), acetone and water and acetone, chloroform, and water (Purchase and Steyn, 1967) were used to extract aflatoxin M_1 from milk powder.

In the first effective method for the determination of Aflatoxin M_1 in fluid milk, methanol and water were used as the extraction solvents (Jacobson *et al*., 1971). This method was modified by others (McKinney, 1972; Stubblefield and Shannon, 1974a), leading to a method suitable for collaborative studies. The method of Stubblefield & Shannon (1974a) involved extraction with acetone and water, precipitation with lead acetate solution to deproteinize the milk, and a defatting step with hexane. TLC with fluorescence detection was used for ultimate separation, detection, and quantification. The collaborative study was successful (Stubblefield & Shannon, 1974b), and the method became an official

method for Aflatoxin M_1 of the AOAC and IUPAC (AOAC official method 974.17, surplussed in 1993).

2.10 Pathogenesis of Aflatoxin

Aflatoxins affect the liver which is very vital organ of detoxification which induced carcinoma. Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and represents

the third cause of mortality among deaths from cancer (Semela and Heim, 2011; EI-Serag and Rudolph, 2007; Yuen *etal*, 2009; Lodato *etal*, 2006; Hainaut and Boyle, 2008; Hai-Xia *etal*, 2011).

There are 600, 000 new cases each year in Asia and sub-Saharan Africa, where populations suffer both from a high prevalence of hepatitis B virus (HBV) infection and largely uncontrolled aflatoxin exposure in food, and more than 200, 000 HCC-related deaths annually in the People's Republic of China alone (Kew, 2002; Wang *etal*, 2002). Aflatoxins may play a causative role in 5%-28% of all global HCC cases (Liu and Wu, 2010). Aflatoxin is a kind of mycotoxins produced by the mold *Aspergillusflavus*, which can be found in legumes, corns, soybeans, rice, milk and cheese throughout their lives. The World Health Organization regarded Aflatoxin B1 (AFB1) as a class 1 carcinogen (Henry *etal*, 2001; López *etal*, 2002). Aflatoxin B1, the most commonly occurring and potent of the aflatoxins is associated with a specific AGG to AGT transversion mutation at codon 249 of the P53 gene inhuman HCC, providing mechanistic support to a causal link between exposure and disease (Goldman and Shields, 2003; Sugimura, 2000; Wild and Montesano, 2009).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

Sliver samples, urine Samples, Breast milk samples, sterile bottles, Urine bottles, HPLC, and distil water.

3.2 Chemicals and Reagents

0.25g NaCl, 40ml distilled water, 0.1 glacial acetate, Methanol, HPLC graded acetonitrite , pH 5.0 sodium acetate buffer.

3.3 Study Area: the study area comprises General Sani Abacha Specialist hospital located in Damaturu, the capital city of Yobe State,North Eastern Nigeria.

Damaturu is a Local Government Area in Yobe State, Nigeria. Its headquarter is in the town of Damaturu, which is also State capital. The postal code of the area is 620. The Local Government Area has an area of 2,366 km² and a population of 88,014 as per the 2006 census. The town of Damaturu is on the A3 highway and has an estimated 2010 population of 44,268. Damaturu is the headquarters of the Damaturu Emirate, at one time part of the Ngazaragamo emirate based in Gaidam. The northeasterly line of equal latitude and longitude passes through the area including 12°00′00″N12°00′00″E in the north (Post Offices- with map of LGA,2012). Fig 3.1 and Fig 3.2 gives the map of Damaturu, Yobe state showing the study area location.

Fig 3.1map of Africa showing Nigeria, Yobe State and Damaturu LGA.

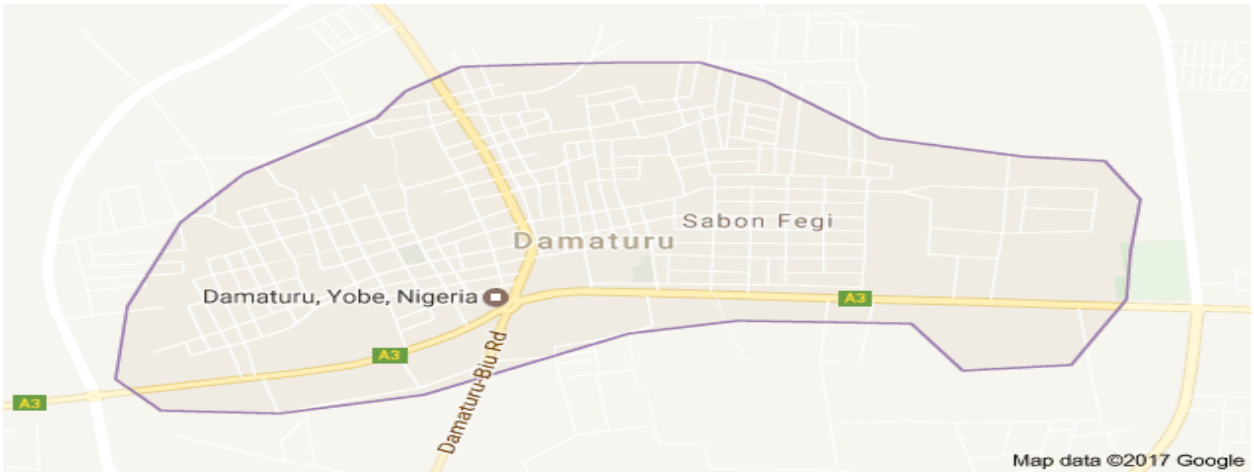


Fig 3.2. The map of DamaturuYobe state showing the study area location

3.4 Ethical Approvaland Parents Consent

Ethical clearance was obtained from the Ethical and Scientific Research Committee of the Ministry of Health, Damaturu Appendix I. A consent form was given to obtain permission from mothers whose babies were on admission. Only those who gave consent were recruited in the study. Appendix II

3.5 Inclusion and Exclusion Criteria

3.5.1Inclusion criteria

All infants on admission in the selected health facility and breast fed20min before collection of samples and who were not administered any antihistamine were included in the study.

3.5.2 Exclusion criteria

Lactating mothers who were on admission, infants with Sjogren' syndrome: an autoimmune disease of the salivary gland, mumps, outpatient, and those on radiotherapy were excluded from the study.

3.6 Sample size was determined using the following equation (Naing*et al.*, 2006):

$$n = \frac{Z^2 pq}{d^2}$$

Where n = sample size;

Z = standard normal distribution at 95% confidence interval**:**

The sample

limit=1.96;

p = prevalence =14.1% (Oluwafemi, 2012);

q = 1-p;

d = absolute desired precision=0.05

$n = (1.96)^2 X 0.141 X (1-0.141) / (0.05)^2$

n = 186

A total of 186 samples were to be collected each from mothers and their infants pair making a total of 372 samples. But for convenience 400 samples were collected in form of one hundred mother infant pair.

3.7 Study Population

The study participants were lactating mothers whose babies were on admission various elements in the selected health facility.

3.8Sample Collection and Handling

Milk and urine samples were taken from lactating mothers as well as saliva and urine samples were collected from their infants of ages 0- 6 months (±2 weeks) before the introduction of any feeding supplements (Tomerak*et al.,* 2011).

3.8.1 Breast milk sample collectionand handling:

Breast milk samples (10ml) were collected aseptically by hand expression into glass tubes; the milk samples were stored at – 20^{o}C. Samples were thawed gradually to 4^{o}C and then centrifuged at 10^{o}C at 15rpm.Aflatoxins are water-soluble; hence, the upper creamy layer was discarded and the lower phase was used for the quantitative test (Adejumo, 2013).

3.8.2 Collection of samples of saliva:

Saliva-Bio Swabs was used for the collection of saliva samples for analysis. The Saliva-Bio Infant's Swab (SIS) (Item No. 5001.08), is recommended for infants under 6 months of age. The back protective package was peel and removed. The SIS was securely held at one end of the SIS device and it was placed under the infants tongue. The swab was held for at about 60-90 seconds at once by resting the swab inside the mouth, till it saturated. The SIS was recap and immediately transported to the laboratory for storage and analysis (www.salimetrics.com).

3.8.3 Urine collection:

Lactating mothers' urine samples and their infants' urine samples were collected separately in plastic urine containers and transferred immediately to the laboratory and stored at -20 ℃ (Manson *et al*., 2015).

3.8.4 Structured questionnaire:

Structuredquestionnairewas design to determine some socio-demographic factors associated with Aflatoxin M1 exposure. To identify kinds of food lactating mothers consumed within 72 hr to associate them to Aflatoxin exposure by considering all the types of food they consumed within the period (Appendix III).

3.8.5Laboratory analysis of samples

3.8.6 Principle of high performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) is basically a highly improved form of column liquid chromatography. Instead of a solvent being allowed to drip through a

column under gravity, it is forced through under high pressures of up to 400 atmospheres. Which makes it much faster (Dhurba, 2015).

Reverse phase HPLC principles:

The stationary phase is nonpolar (hydrophobic) in nature, while the mobile phase is a polar liquid, such as mixtures of water and methanol or acetonitrite. It works on the principle of hydrophobic interactions hence the more non-polar the material is, the longer it will be retained (Dhurba, 2015).

3.8.7. Preparation of breast milk samples for high performance liquid chromatography (HPLC)

Aflatoxin M1 was extracted from the breast milk according to the International Standard Organization (1998) with slight modifications. Adejumo (2013) was adopted. Forty (40) ml distilled water was added to 10 ml of breast milk in a100ml beaker followed by the addition of 0.25g NaCl and the solution was mixed properly. The mixture was then centrifuged at 4000 rpm for 10 min. After which the skim portion (bottom layer) was filtered through a glass microfiber filter and 8ml of the filtrate passed through an Aflatestimmunoaffinity column (VICAM,Watertown, MA, USA)fitted on a solid phase manifold (Superlco,Bellefonte, PA)at a flow rate of 1–2drop/s. Eight (8)millilitres of methanol: water (10:90)was used to wash the column and the bound aflatoxin M1 eluted with 3ml of methanol into an amber vial at the rate of 1–2 drop/s. The eluate was dried in acentrifugal evaporator vacuum centrifuge (Savant Instruments Inc., Farmingdale, NY, USA)reconstituted in 200 µl of methanol and 50 µl portion injected into HPLC for analysis.

3.8.8 Preparation of urine samples for high performance liquid chromatography (HPLC)

The extraction and purification of urine samples for AflatoxinM1 determination were performed as recommended by Kussak *et al.*,(1995). Exactly 30 ml from the urine sample was filtered through a glass microfibre filter paper (Whatman Schleider & Schuell, Maidstone, England, product number 934-AH). Later, 20 ml of filtered extract was transferred to a 50 ml capacity vial and 20 ml of sodium acetate buffer (pH 5.0) was added. The pH of the mixture was measured and corrected to 5.0 using an appropriate volume of a 0.1 M glacial acetic acid solution whenever necessary. The mixture was directly passed through an immunoaffinity column (Neocolumn, Neogem Europe, UK) at a flow rate of approximately 1.0–1.5 ml min^{-1}. After adding the mixture the column was washed with 40 ml of ultrapure water (Milli Q, Millipore, and Bedford, MA, USA). The column was dried by applying positive pressure with a syringe and bound AFM1 was eluted with 2.0 ml of HPLC-methanol which was recovered in a 4 ml vial previously treated with acid. The eluate was evaporated under nitrogen gas and reconstituted with 500 µl of the mobile phase before liquid chromatography analysis. Detection and quantification of sample extracts were performed by high-performance-liquid chromatography (HPLC) with a liquid chromatography system equipped with a LC-10AT Shimadzu pump (Kyoto, Japan), a Shimadzu RF-10AXL fluorescence detector (excitation 365 nm and emission 460 nm), an injection volume of 100µl, and a reverse phase column (250- 4.6mm, particle size of 3 µm) and pre column (Synergi Fusion, Phenomenex Inc., Torrance,CA, USA) kept at room temperature. The mobile phase consisted of an isocratic mixture of water and acetonitrile at a volume ratio of 75:25 and a

flow rate of 1.0 ml min^{-1}. A calibration curve was prepared using standard AFM1 solutions in mobile phase at concentrations of 0.005, 0.01, 0.02, and 0.03 ng ml^{-1} The standard obtained (Sigma, St. Louis, MO, USA, and product code 6428, 10 lg) as purified crystalline AFM1 was dissolved in HPLC-grade acetonitrite and its concentration was determined by spectrophotometer according to the method ofTrucksess (2005).

3.8.9 Preparation of saliva samples for high performance liquid chromatography (HPLC)

The extraction and purification of saliva samples for AFM1 determination were performed. A 30 ml volume from the saliva sample was filtered through a glass microfibre filterpaper (Whatman Schleider, Schuell, Maidstone, England, product number 934-AH). Later, 20 ml of filtered extract was transferred to a 50 ml capacity vial and 20 ml of sodium acetate buffer (pH 5.0)was added. The pH of the mixture was measured and corrected to 5.0 using an appropriate volume of a 0.1 M glacial acetic acid solution whenever necessary (Kussak *et al.*,1995).

3.8.10 High performance liquid Chromatography (HPLC), Analysis for Aflatoxin M1 High performance liquid Chromatography (HPLC), was used to analyzed the breast milk, saliva, and urine samples, (Barbieri*et al.*, 1994).

The concentration of AflatoxinM1 in breast milk, saliva and urine samples were estimated by HPLC (Shimadzu, Japan) configured with LC-10AD pumps, coupled with tungsten detector RF-10Axl. Excitation and emission wavelengths were set at 350 and 440 nm respectively. The stationary phase was a Gemini Column (C18 TMS end-capping, 3 lm particle size, 150-4.60 mm, pore size 110 Å, Phenomenex, USA). The

mobile phase was isocratic mixture of methanol/acetonitrite/water (25:25:50 v/v/v), with a flow rate of 1ml/min and chromatographic run time of 10 min. The values obtained for recoveries and relative standard deviations of the methods of analysis were in agreement with Commission Regulation (EC) No. 401/2006 for methods of analysis of mycotoxins in foodstuffs (European Union Commission, 2006).

An injector with 50 µl loop was used for the determination of AFM1. A calibration curve was constructed for AF M1 using different levels of toxin concentrations with an average of 10 consecutive automated injections of standard solutions of AF M1 purchased from SUPELCO solutions within^{TM} USA. Involving series of dilutions (Adejumo ,2013).

3.9 Analytical Performance of HPLC

The limit of detection (LOD) for AFM1 was estimated at 0.01 µg/mL and the limit of quantification (LOQ) was 0.05 µg/mL. The linearity of the curve was 0.01–0.05 µg/mL. The calibration curve for AFM1 had a linear equation of y = 4147.x - 230.3. Appendix 4 gives the calibration curve with a correlation coefficient $R^2 = 0.993$ and retention time of 10.0 min.

3.10 Socio- Demographic and Food Consumptions Determination

The socio- demographic and food consumption were determined using a structured questionnaire as shown in Appendix III

3.11 Statistical Analysis of the Data

The Statistical Package for Social Science program (SPSS, Chicago, Illinois, USA) version 21 was used for analysis of data. Data was summarized as mean, standard

deviation, for the analysis of the difference in quantitative data between the two groups. Odd Ratios were determined in breast milk samples and urine samples of infants, and also saliva samples and urine samples of infants. The X^2-test was used for the analysis of qualitative data. *P value* was considered significant if it was less than 0.05. Simple linear correlation (Pearson's correlation for quantitative data Tumerak, 2011).

CHAPTER FOUR

4.0 RESULT

4.1 Occurrence and Concentration of AflatoxinM1 in Lactating Mothers

From the result obtained the occurrence rate and concentration of Aflatoxin M1 in lactating mothers' breast milk and urine are summarized as follows:

The occurrence of AFM1 in 100 lactating mothers and their infants as presented in Table 4.1 show higher occurrence of 93.3% of AFM1 was recorded in urine samples of lactating mothers when compared with 82% in their breast milk. There was no statistically significant association in AFM1 occurrence between breast milk and urine of lactating mothers (OR=0.987;χ^2 = 1.765; p= 0.457).

The result also reveals the occurrence of AFM1 among infants with 68% in the urine of infants when compared with63% in their saliva. There was no significant association between the occurrence of AFM1 in urine and saliva (OR=1.234, χ^2 =3.456; p=0.143)

Percentage occurrence of AFM1 in breast milk and urine samples of lactating mother in relation to selected socio-demographic factors can be observed in table 4.2.The levels of contamination of breast milk samples from 100 mothers,shows occupation as factor, unemployed mothers recorded higher occurrence of 83.9%. There was statistically significant association between the occurrence of AFM1 among employed and unemployed participants. (OR=2.312,χ^2 =12.687; p=0.045). Age brackets 18-25 showed higher occurrences of 83.6%, and there was significant association between the ages of participants and the occurrence of AFM1 (χ^2 =8.121; p=0.034). The occurrence of AFM1

was found to be highest among those with informal education with 97.4% and there was statistically significant association between their educational status and aflatoxin occurrence (χ^2 =7.065; p=0.05).

The percentage occurrence of AFM1 in the urine of lactating mothers in relation to the Socio-demographic factors (table 4.2). AFM1 occurrence was higher among unemployed mothers with 100% occurrence and significant statistical association (OR=2.564, χ^2 =20.451; p=0.001). Age had significant association with AFM1 occurrence. Mothers between 42 and above had 100% positively (χ^2 =5.671; p=0.047). Education status also influence AFM1 occurrence. Mothers with informal education had 100% exposure and there was significant association between educational status and exposure. (χ^2 =8.756; p=0.048).

4.2 Occurrence and Concentration of AflatoxinM1 in infants

The percentage occurrence of AFM1 in infants' saliva and urine samples in relation to their age in months and sex in table 4.3 shows that 95% of the infants were exposed within the ages of 5-6 months (χ^2 =10.08; p=0.032). Ages 3-4 had 88% (χ2=3.541; p=0.089); males had 78.1% but there was no significant statistical association between the sexes (OR= 0,611;χ^2 =0.611; p=0.113).

Table 4.4 shows the occurrence of AFM1 in urine samples of lactating mothers with respect to the food they consumed within 72hrs prior to sample collection. Around 97.1% of the mothers consumed milk (OR =2.141 χ^2 =8.247; p=0.046); 100% of the mothers consumed Meat, corn meal was consumed by the mother 93.4% (OR = 0.654,χ^2 =10.23; p=0.014); Date was consumed by 93.3% (OR =2.789 χ^2 =8.911; p=0.043); while

'brabisko/biski' (local food) 30.6% (OR =1.154 χ^2 =11.654; p=0.048). From the results 97.4% of the mothers did not consume imported rice (OR =1.010 χ^2 =7.521; p=0.063); but 93.4% of them consumed native rice (OR =2.500χ^2 =9.112; p=0.050).

The concentration of AFM1 in 100 infants' saliva samples according to their age and sex in table 4.6 shows the concentration of 0.08µg/L was detected in ages 5-6 and 0.06µg/L was detected among the male samples. Urine samples were observed among ages 5-6 months to contain 0.07µg/L AFM1 while age ≤ 2 months had least concentration of 0.04µg/L.

4.3 Some socio-demographic factors associated with AFM1 exposure

Some of the socio-demographic factors associated with aflatoxin M1 exposure in tables 4.2 and 4.5 are occupation, Age, and educational level showing statistical significance with p≤0.05. The urine sample shows a very strong statistical association in respect to occupational status (p=0.001). However, ages 34-41had the least percentage exposure in their breast milk (60%).But 18-25 and 34-41 had more aflatoxin M1 both in breast milk and urine samples (0.07ug/L)

4.4. Survey of Types of Food Lactating Mothers Consume To Associate Them to Aflatoxin Exposure

The category of food lactating mothers consume in table 4.4 within 72hrs shows that those that consume meat, milk, corn meal, date and native rice had the highest percentage exposure to aflatoxin: 100%, 97.1%, 93.4%, 93.3% and 93.4% respectively.However those that consume local food had lower percentage of 30.6%.

Table 4. 1 Occurrences of AFM1 among Lactating Mothers and Their Infants in Damaturu Yobe, Nigeria

Samples Mothers	**No examined**	**No. positive (%)**	**Odds ratio**	**Chi square**	**p-value**
Breast milk	100	82(82.0)	0.987(0.678-1.654)	1.765	0.457
Urine	100	93(93.0)			
Infants					
Saliva	100	63(63.0)	1.234(0.789-2.102)	3.456	0.143
Urine	100	68(68.0)			

Table 4 .2 Percentage Occurrence of AFM1 in Breast Milk and Urine Samples of Lactating Mothers in Relation to Selected Socio-Demographic Factors.

Samples /Category	**No examined N=100**	**No. positive**	**(%) occurrence**	**p-value**
BreastMilk				
Occupation				
Employed	13	9	69.2	0.045
Unemployed	87	73	83.9	
Age(yrs)				
18-25	55	46	83.6	
26-33	39	32	82.1	0.034
34-41	5	3	60.0	
42above	1	1	100	
Educationallevel				
Informal	39	38	97.4	
Islamic	43	30	69.7	0.05
Primary	12	9	75.0	
Secondary	6	5	83.3	
Urine				
Occupation				
Employed	13	6	46.1	0.001
Unemployed	87	87	100.0	
Age (yrs)				
18-25	55	51	92.7	
26-33	39	36	92.3	0.047
34-41	5	5	100.0	
42above	1	1	100.0	
Educationallevel				
Informal	39	39	100.0	
Islamic	43	37	86.0	0.048
Primary	12	12	100.0	
Secondary	6	5	83.3	

Table 4.3 Percentage Occurrence of AFM1 in Infants' Saliva and Urine Samples in Relation to their Age and Sex

Samples	**No examined N=100**	**No. positive**	**(%)**	**Chi square**	**p-value**
Saliva		8	25.0		
Age (months)					
≤ 2	32	19	76.0	10.008	0.032
3-4	25	41	95.3		
5-6	43				
Sex		26	81.3		
Male	32	42	61.7	8.432	0.043
Female	68				
Urine					
Age (months)		10	31.2		
≤ 2	32	22	88.0	3.541	0.089
3-4	25	31	72.9		
5-6	43				
Sex		25	78.1		
Male	32	38	55.9	1.127	0.113
Female	68				

Table 4.4 Occurrence of AFM1 in Urine Samples of Mothers with the Types of Food the Consumed Within 72 Hrs Prior to Sample Collection.

Consumed foods in 72 hrs	**No examined N=100**	**No. positive (%)**	**Odds ratio**	**Chi square**	**p-value**
Milk					
Yes	67	65(97.1)	2.141(1.911-3.711)	8.247	0.046
No	33	28(84.1)			
Meat					
Yes	43	43(100.0)	1.145(0.789-1.654)	3.123	0.081
No	57	50(87.7)			
Cornmeal					
Yes	79	74(93.4)	0.654(1.112-1763)	10.231	0.014
No	21	19(90.5)			
Date					
Yes	45	42(93.3)	2.789(1.002-3.456)	8.911	0.043
No	55	51(92.7)			
Brabisko/Biski(local food)					
Yes	36	11(30.6)	1.156(0.711-1.811)	11.654	0.048
No	64	60(93.8)			
ImportedRice					
Yes	22	17(77.3)	1.010(1.151-1.863)	7.521	0.063
No	78	76(97.4)			
NativeRice					
Yes	63	59(93.4)	2.500(1.001-3.457)	9.112	0.050
No	37	34(91.9)			

4.5Correlation between AflatoxinM1inMothers and their Infants.

The table 4.7 shows the correlation between lactating mothers' breast milk samples and their infants' saliva samples. AFM1 secretions (r=1.475 and p=0.039) in mother's milk and infants saliva has statistically significant relationship. The correlation between lactating mothers' urine and their infant's saliva for AFM1 detection was not statistically significant. (r=0.325, p=0.089). The correlation between the detection of AFM1 in urine of lactating mothers and their infants shows no statistical significant positive relationship ($p>0.05$)

Table 4.5: Concentration of AFM1 in Breast Milk and Urine Samples of Lactating Mothers According to Selected Socio-Demographic Factors

Socio-demographic factors /samples	Sample tested No. N=100	Positive samples No.	Percentage of positive	Mean AFM1 (µg/L)
Milk Sample				
Occupational Position Of Mothers				
Employed	13	09	69.2	0.06 (±0.02)
Unemployed	87	73	83.9	0.07 (±0.01)
Age				
18-25	55	46	83.6	0.07(±0.03)
26-33	39	32	82.1	0.05(±0.02)
34-41	05	03	60.0	0.07(±0.01)
42-above	01	01	100.0	0.04(±0.03)
Educational Level				
Informal education	39	38	97.4	0.07(±0.02)
Islamic education	43	30	69.8	0.06(±0.01)
Primary school cert.	12	09	75.0	0.07(±0.03)
	06	05	83.3	0.06(±0.02)
Urine Sample				
Occupational Position of Mothers				
Employed	13	6	46.1	0.04(±0.01)
Unemployed	87	87	100.0	0.05(±0.02)
Age				
18-25	55	51	92.7	0.05(±0.02)
26-33	39	36	92.3	0.04(±0.02)
34-41	05	05	100.0	0.05(±0.02)
42-above	01	01	100.0	0.04(±0.01)
Education Level				
Informal education	39	39	97.4	0.05(±0.02)
Islamic education	43	37	86.0	0.04(±0.02)
Primary school cert.	12	12	100.0	0.04(±0.01)
Secondary school cert.	06	05	83.3	0.04(±0.02)

Table 4.6: Concentration of AFM1 in Infants Saliva and Urine Samples According to Age and Sex.

Infant Age/Sex	**Sample tested No. N=100**	**Positive samples No.**	**Percentage of positive**	**Mean AFM1 (µg/L)**
Saliva				
Infant Age (month)				
≤ 2	32	8	25.0	0.04 (±0.01)
3-4	25	19	76.0	0.07 (±0.03)
5-6	43	41	95.0	0.08 (±0.05)
Infants Sex				
Male	32	26	81.3	0.06 (±0.02)
Female	68	42	61.8	0.05 (±0.02)
Urine				
Infant Age (month)				
≤2	32	10	31.3	0.05 (±0.02)
3-4	25	22	88.0	0.06 (±0.03)
5-6	43	31	72.1	0.07(±0.02)
Infants Sex				
Male	32	25	78.3	0.06 (±0.01)
Female	68	38	55.9	0.05(±0.03)

Table 4.7 Correlationsbetween Lactating Mothers' Breast Milk AFM1 Concentration and Infant Saliva AFM1 Concentration

Sample	**Mean value µg/L**	**Pearson correlation coefficient**	**P –value**	**Deduction**
Breast milk and Saliva of infant	0.057±0.21 0.049±0.13	0.975	0.039	Strong Positive correlation and significant
Urine of mothers And Saliva of infant	0.069±0.35 0.049±0.13	0.325	0.089	Weak correlation and not significant
Urine of mother And Urine of infant	0.069±0.21 0.055±0.49	0.711	0.12	Positive correlation

CHAPTER FIVE

5.0DISCUSSION

The occurrence of AFM1 amonglactating mothers breast milk samples is at 82% frequency, while 93% of urine samples contained AFM1. This could be as a result of dietary exposure of the screened mothers to AFMB1 which is metabolized to AFM1 and secreted in the breast milk and also excreted in the urine. Coincidentally, another independent screening by Adejumo, *et al* (2013) who also reported occurrence of AFM1in 82% of breast milk samples in Ogun state, Nigeria. Abdulrazzaq *et al.,* (2003) and Sadeghi *et al.* (2009) in United Arab Emirate and Iran respectively reported 99.5% and 98.1% frequencies. The percentage occurrence in this study is higher than that reported in of Sierra Leone, Cameroun and Egypt, 31%, 4.8%, and 56% respectively (Jonsyn *et al*, 1995; Tchana *et al*., 2010; Polychronaki *et al*., 2007). This could be as a result of differences in the type of food consumed, storage condition of foods and lifestyle of the people as well as level of contamination with the secreting *Aspergillus species.*

Urinary excretion of AFM1 among lactating mothers showed that 100% of unemployed mothers had tested for the toxin in their urine compared to the employed mothers. This suggests that the unemployed mothers are better exposed through their diets. One hundred percent of people with informal education and primary school leavers were exposed to AFM1;the educational qualification of lactating mothers is significantly associated with secretion of AFM1 in breast milk, the highest % secretions was people with informal education. This disagrees with Adejomo *et al*., (2013), that reported that the primary school leavers had the highest level of AFM1 in there breast milk samples.

The exposure based on excretion of AFM1 in urine disagrees with Mason *et al.*, (2015), that recorded that tertiary educational level had highest exposure of AFM1.

Concentration of AFM1 among lactating mothers' breast milk in relation to socio-demographic factors expressed the highest concentration among unemployed and age bracket 18-25 and 34-41 with 0.07µg/l which could be that the employed mothers were more careful with quality of food they consumed while the unemployed mothers may concentrate on the quantity of food they took. In the case of excretion in urine 0.05µg/l AFM1 was tested among unemployed mothers with lower concentration among the age of 42 and above having 0.04 µg/l this may be due to the elder mothers may be more careful on quality of food consumed. Looking at educational background within 72hrs those with informal education had highest concentration of 0.05µg/l under this category they tend to be more likely less careful. was within the limits gotten by Adejumo *et al* (2013) but lower in Oluwafemi (2012).

Among theinfants tested, 63% secreted AFM1 in their saliva while 68% excreted AFM1 in their urine. These infants were exclusively breast fed under baby friendly programme of the government, that means the infant were fed exclusively with breast milk from their mothers during the study .However, since Infants have lower detoxification capacity, rapid growth rate and higher in takes of milk continuous exposure to AFM1 through breast milk will pose danger to the infants (Jafarian-Dehkordi *et al*., 2013). Excretion of AFM1 in the urine among infants that suffered kwashiorkor as reported by Onyemelukwe(2012) was noted in less than 84.3%of such infants suggests that infants who are malnourished may be more amendable to AFM1 production.

The occurrence of AFM1 in infant saliva is significantly related to age and sex, from this work 95.3% of infants aged 5-6 months secreted AFM1in their saliva while only 25.0% of infants aged 2months and less did so. It is obvious that at age 5-6 the infants can start eating, picking dirt apart from the breast milk. The excretion of AFM1 in urine indicated that male had higher percentage of 78.1% exposure and secretion of 81.3% in their saliva; this could be explained that male infants probably suck more breast milk than the females.

Occurrence of AFM1 in infant's urine in relation to their age and sex was found to be 88% between the ages of 3-4 months with no statistical significance. This agrees with the study reported in Brazil that age and sex was not significant to AFM1 excretion in urine of infant (Giolo *et al.*2012).

The concentration of AFM1 found among the infants showed short time exposure of aflatoxin in the infants which is significant, 0.08µg/L concentration tested higher among 5-6 this could be that at this age the infant tend to feed more than the lower ages which predisposed them to AFM1. Urine samples were also observed among 5-6 to have 0.07 µg/L and ≤ 2 had 0.04 µg/L which were lower than in older infants and this may be they consumed lesser volume of milk than the others. This result can be compared with other researchers that there is a relationship between food intake and urinary excretion of AFM1 (Mason *et al.*, 2015). Redzwan *et al* (2012) reported that there was significant relationship between consumption of AFM1 in milk and excretion in the urine.

Concentration of AFM1 in infant saliva according to age and sex: 0.08µg/L was observed among ages 5-6 months which is considered higher than the recommendation by Codex Alimentarius (1995), of 0.05µg for milk product.

AFM1 levels in contaminated breast milk samples from lactating mothers in relation to some socio-demographic factors showed that 83.9% unemployed mothers were exposed, in contrast to 69.2% exposure by employed mothers. This could mean that employed mothers are more enlightened about proper and healthy food consumption practices than the unemployed mothers. Also 83.6% of mothers between the ages of 18-25 had AFM1 in their breast milk samples. As young, active mothers, they are probably exposed to high levels of AFM1precursors. Educational level of mothers also significantly influences contamination of breast milk with AFM1. 97% of the mothers with informal education had AFM1in their breast milk samples while those with Islamic education had 69.7%. This can be suggested that the levels of education played significant role on exposure to AFM1 precursors among lactating mothers. Some socio-demographic factors are significantly associated with the occurrence of AFM1 among lactating mothers. This also agrees with Mason *et al.* (2015).

AFM1 excreted in urine of lactating mothers within 72hrs of food consumption, showed 84% of mothers that took milk, AFM1was not detected, meat: 100% were exposed to AFM1, corn meal: 93.4% exposed to AFM1, also, date: 93%, 'Brabisko or Biski': 30.6%, imported rice: 77.7%, native rice: 93.4% this could be that the process of food handling contribute to the contamination and invariable contributed to infants exposure to AFM1 in breast milk. Therefore, their food consumption and the type of food were associated with AFM1 exposure by the infants. Although meat, and imported rice showed no significance association with AFM1 exposure. However, Romero *et al.,* (2010) investigated the presence of AFM1 in Brazilian population and noted that it is not dependent on corn, peanut and milk consumption alone. From this study one of the major

risk factors associated with exposure to AFM1 is the type of food taken by lactating mothers which agrees with Iha *et al.,* (2014).

From the study, there was a positive correlation between secretions of AFM1 in the breast milk of lactating mothers and the secretions of AFM1 in the saliva of their infants. The implication is that the more the mothers were exposed, the more the infants are equally exposed. However, this continuous exposure to the toxin from the saliva of the infants may be absorbed from the gastrointestinal tract in a cycle as reported by Caloni, *et al.*, (2006). This toxin my stay long in the systems of the infants.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The level of AFM1 was assessed in the breast milk secretions and excretions of lactating mothers breast milk whose infants were on admission in General Sani Abachia Specialist Hospital Damaturu and the occurrence was found to be considerably high. However the following conclusions were made:

The percentage occurrence of lactating mothers' breast milk and urine were 82% and 93% respectively. While the mean concentration of AFM1in breast milk and urine samples were found to be 0.057µg/L and 0.069µ/Lrespectively.

The percentage occurrences of AFM1among infants were 63% and 68% in saliva and urine respectively. While, the concentration in saliva and urine samples were found to have mean values of 0.049µg/L and 0.055µg/L. There was a positive correlation between lactating mothers breast milk AFM1 and the secretion of AFM1 in the infant's saliva with p-value 0.0039, but no positive statistical correlation with secretion their urine.

Considering unemployment as one of the socio-demographic factors associated with exposure of AFM1 had the highest exposure and age brackets 18-33 but the least was 42 years and above.

Food consumptionsuch as milk, corn food, date, native rice; were claimed to serve as the sources ofAflatoxin exposure among lactating mothers.

It should be noted that, the concentration of AFM1 in all the breast milk samples used were higher than the acceptable tolerance level of 0.05µg/L for infants milk by the European Communities and Codex Alimentarius. This posses a concern that infants that were on admission in the selected facility where exposed to AFM1.This implies that AFM1 has the potential to be a public health problem in Damaturu.

6.2 Recommendations

Based on the outcome of this study, the following recommendations can be made

i. There is the need to continue monitoring the levels of AFM1 in lactating mothers to reduce early infancy exposure to aflatoxin.
ii. Continuous breast feeding of infants is still the best and recommended way for infant nutrition. However emphasis should be placed on the quality of food mothers need to take for the wellbeing of their infants.
iii. Comprehensive plan to train mothers to avoid eating highly contaminated food is a practical and useful methods for aflatoxin exposure, control measures can be adopted to cheek maternal and child Aflatoxin exposure.

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APPENDIX I

Ethical Clearance

SECRET

YOBE STATE GOVERNMENT OF NIGERIA

MINISTRY OF HEALTH & HUMAN SERVICES

Near General Sani Abacha
Specialist Hospital,
Along Gujba Road, P.M.B. 1047,
Damaturu, Yobe State.

Our Ref: MOH/GEN/474/Vol.I Date: 2nd December,2015

Alegbe Sunday David,
Department of Microbiology,
Ahmadu Bello University,
Zaria.

Dear A.D. Sunday

RE: ETHICAL CLEARANCE/APPROVAL TO CONDUCT RESEARCH ON THE EFFECT OF AFLATOXIN M1 LEVELS FROM LACTATING MOTHERS ON INFANTS SALIVARY IgA

Further to your application in respect of the above research proposal which you intended to carry out using 200 breast milk and infant saliva samples from consented volunteers in Specialist Hospital Damaturu and General Hospital Potiskum. The Yobe State Health Research and Ethics Committee (YOHREC) considered the proposal and noted as a population-based study.

In view of this, an exempt ethical approval is hereby granted to conduct the research.

However, the approval is subject to periodic oversight supervision by the committee members and the Findings of the study should be shared with SMOH, SPHCMB and HMB.

Thank you in anticipation, please.

Muhammad Liman
Secretary
For: Hon. Commissioner

All Correspondence to the Office of the Honourable Commissioner

APPENDIX II

Informed Consent Form (ICF)

Serial No……. Hospital No: …… Age…….. Phone No ……

This informed consent form is basically for Lactating mothers whose children are on admission in selected Hospitals in Yobe State. We are inviting you to participate in this research work titled **ASSESSING THE LEVELS OF AFLATOXIN M1 AMONG LACTATING MOTHERS AND THEIR INFANTS IN DAMATURU, YOBE STATE NIGERRIA.**

Breast milk is a major nutrient for infants, feeding of infants with safe milk is essential; the building of infant immunity will depend on the intake of its major nutrient. Aflatoxin M1 (AFM1) may exert interactions affecting immunoreactivity in infants.

The research will involve the collection of breast milk from participants in a sterile tube by gently hand expression by the mothers into the tubes, the sample will be collected three times (Morning, Afternoon and Evening). The saliva of their respective infant will be collected in non invasive procedure by bio-infant swab from the saliva pool. Individual data will be treated with strict confidentiality.

Participation is strictly voluntary and refusal to participate in the research will not in any way affect your right and benefit in this clinic/ hospital.

CERTIFICATE OF CONSENT

I _____ of _____ do hereby consent to participate in this study. The full procedures of the test/study have been explained to me by the investigator. I understand that my breast milk and my child's saliva will be analyzed. I therefore give this consent voluntarily without being subjected to any pressure.

Name of participant_____

Signature/Thumbprint _____
Date_____

Statement by witness

I have witness the accurate reading of the consent form and details explanation of the study to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Name of witness_____

Signature of witness/ Thumbprint _____

Date _____

Name of researcher _____

Signature _____

Date _____

APPENDIX III

QUESTIONNAIRE

ASSESSMENT OF AFLATOXIN M1 AMONG LACTATING MOTHERS AND THEIR INFANTS IN DAMATURU YOBE STATE, NIGERIA.

This questionnaire requires information about the aforementioned study. We request you fill it to the best of fact. You should be rest assured that the information provided will be treated with utmost confidentiality. Please tick where necessary. Thank you.

Age:_____

Educational status (a) Primary [ ] (b) Secondary [ ] (c) Tertiary [ ] (d) Arabic/Islamic [ ]

(e) Informal [ ]Marital status (a) married [ ] (b) single [ ] (c) divorced [ ]

Occupation _____Place work_____

Place of residence _____

How long your Baby has been on admission? _____

How old is your baby: in Months

0-1 [ ] 2 [ ] 3 [ ] 4 [ ] 5 [ ] 6 [ ] above 6 [ ]

Sex of your baby: Female [ ] Male [ ]

Answer yes or no

Are you currently under any treatment? Yes [ ] No [ ]

Is your baby always on admission? Yes [ ] No [ ]

Does your baby breast feed normally? Yes [ ] No [ ]

Is your baby undergoing baby friendly? Yes [ ] No [ ]

Is your baby undergoing any radiotherapy? Yes [No [ ]

Do you have any autoimmune disease? Yes [ ] No [ ]

Do you normally give your baby multivitamin? Yes [ ] No [ ]

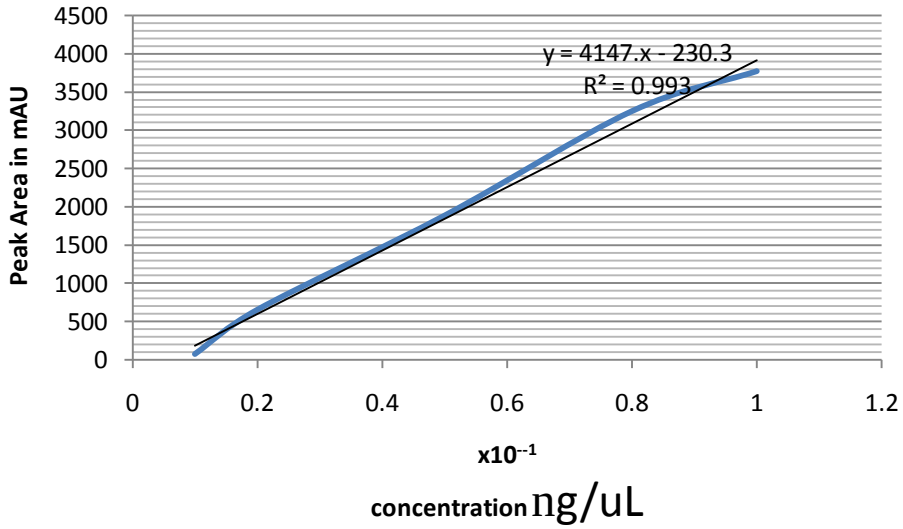
Is your baby on any cold/ cough syrups? Yes [ ] No [ ]

Mention the kinds of food / fruit you frequently eat including yesterday

_____

APPENDIX IV

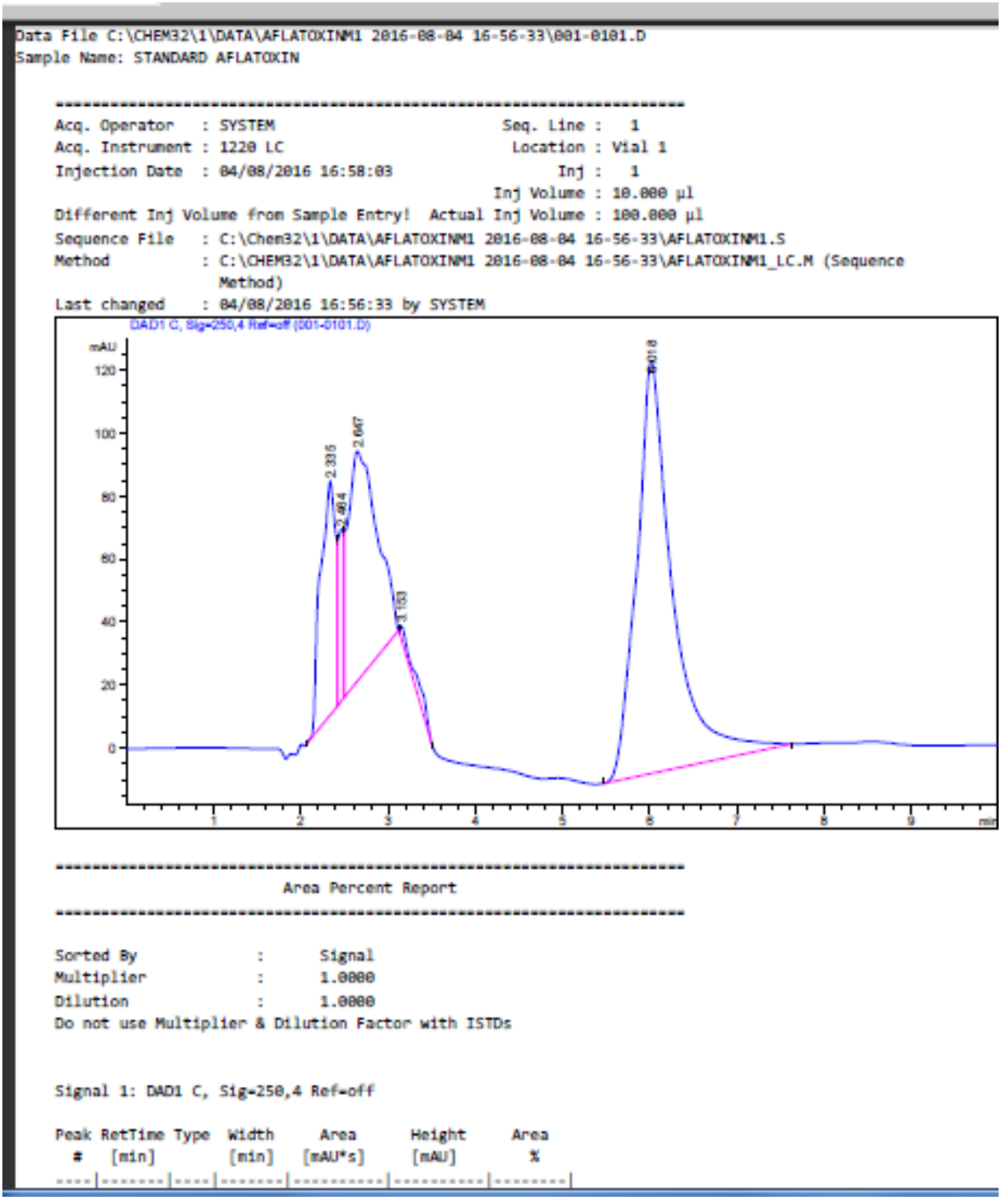
Standard Calibration curve for Aflatoxin M1 purchase from SUPELCO solutions USA



Key: mAU= milli adsorbent unit

APPENDIX V

HPLC sampled Chromatographs

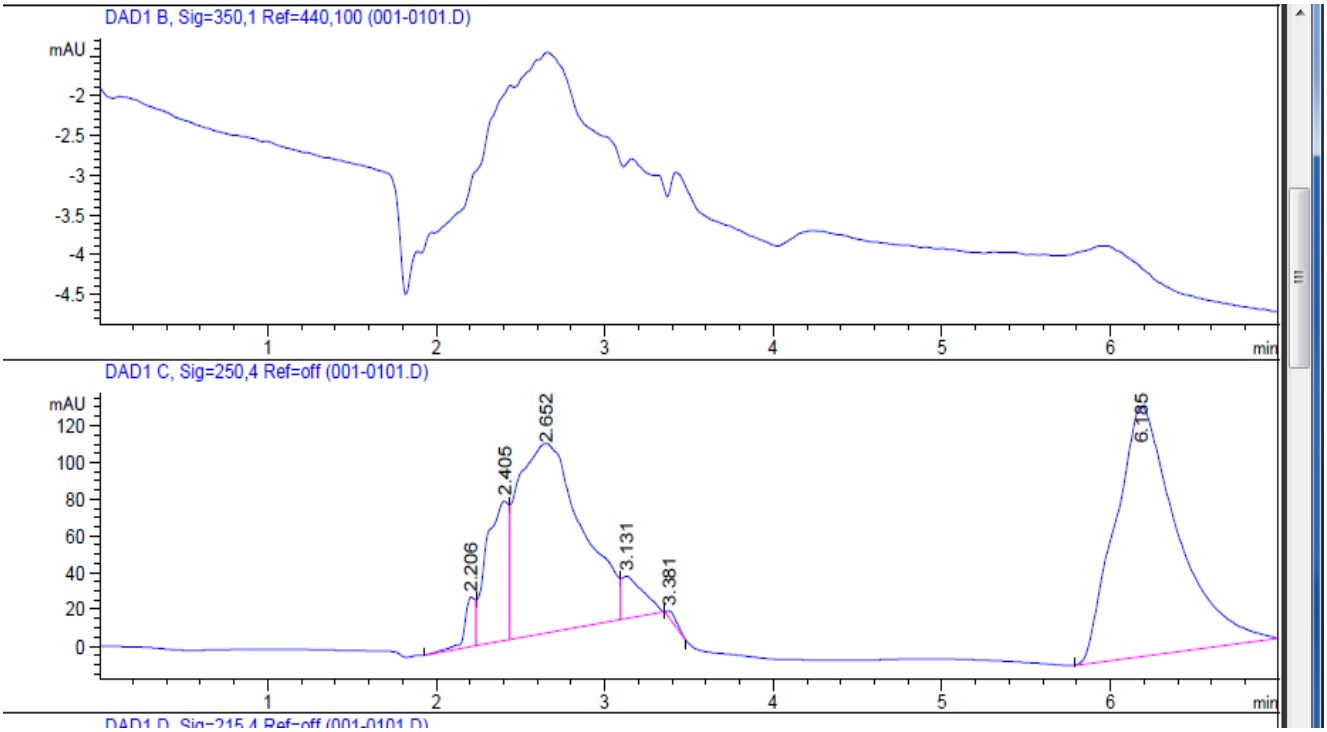


```
Data File C:\CHEM32\1\DATA\AFLATOXINM1 2016-08-04 16-56-33\001-0101.D
Sample Name: STANDARD AFLATOXIN

-------------------------------------------------------------------
Acq. Operator   : SYSTEM                        Seq. Line :   1
Acq. Instrument : 1220 LC                       Location : Vial 1
Injection Date  : 04/08/2016 16:58:03                Inj :   1
                                               Inj Volume : 10.000 µl
Different Inj Volume from Sample Entry!  Actual Inj Volume : 100.000 µl
Sequence File   : C:\Chem32\1\DATA\AFLATOXINM1 2016-08-04 16-56-33\AFLATOXINM1.S
Method          : C:\CHEM32\1\DATA\AFLATOXINM1 2016-08-04 16-56-33\AFLATOXINM1_LC.M (Sequence
                  Method)
Last changed    : 04/08/2016 16:56:33 by SYSTEM
```

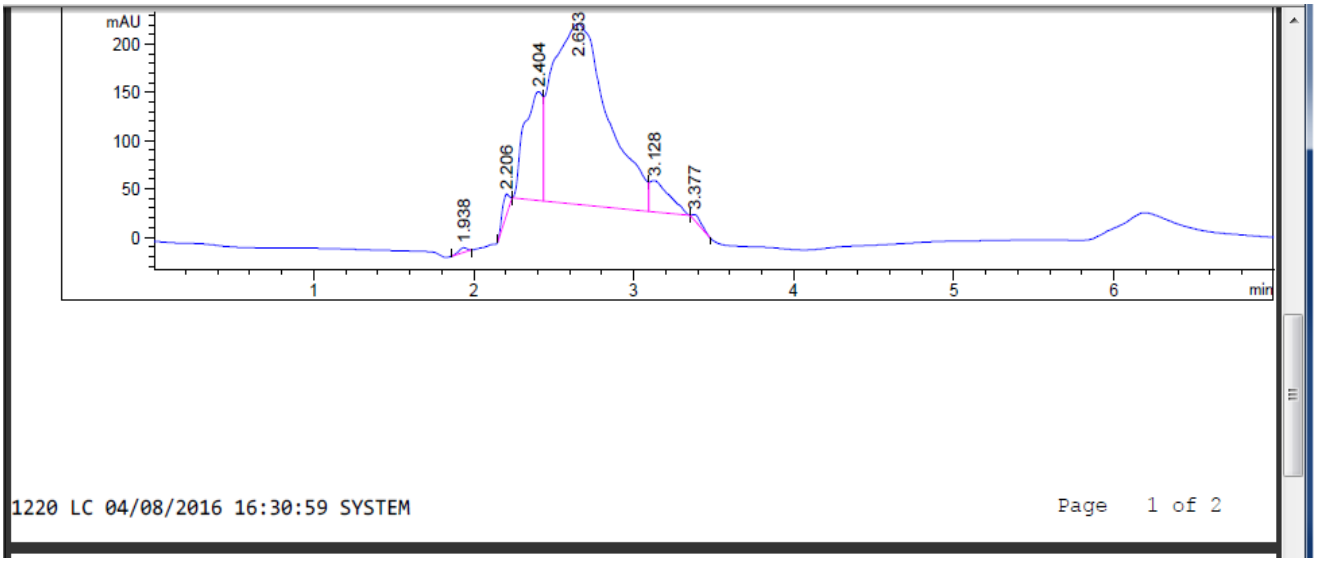


```
-------------------------------------------------------------------
                        Area Percent Report
-------------------------------------------------------------------

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Do not use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 C, Sig=250,4 Ref=off

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
```

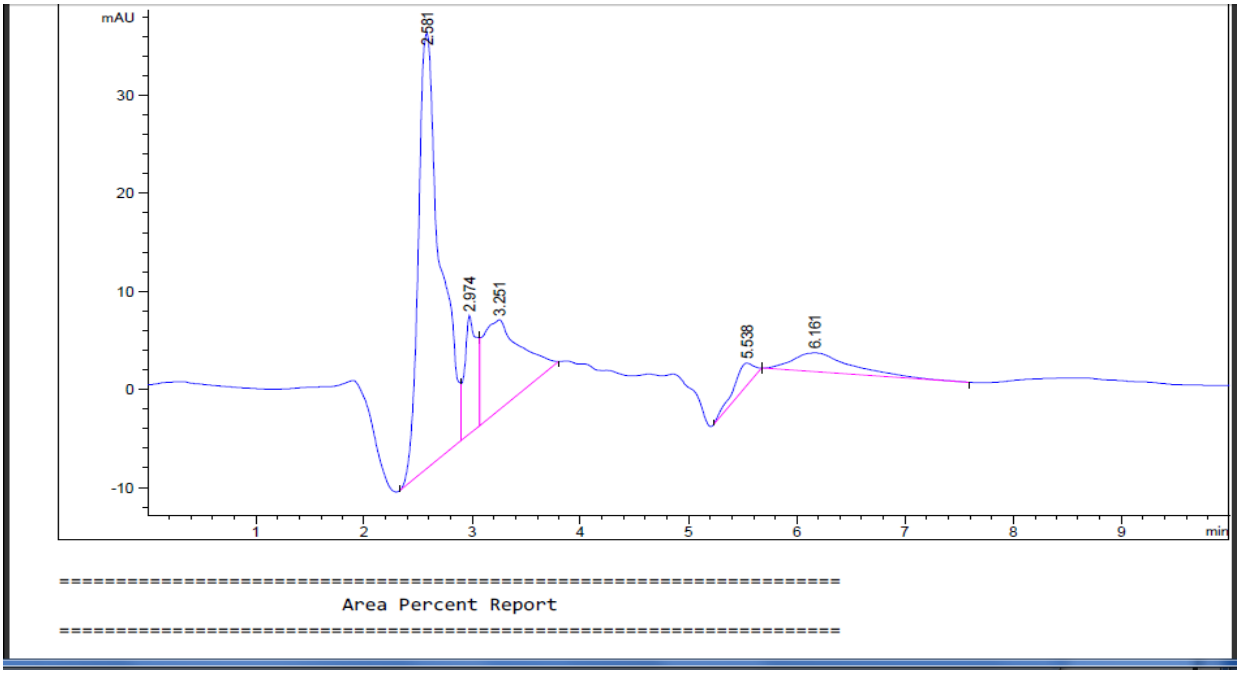
Standard Aflatoxin M1



Chromatograph of urine samples



Chromatograph of infants saliva



Chromatograph of breast milk

8