Journal of
Health, Medicine and Nursing
(JHMN)

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IPRJB
INTERNATIONAL PEER REVIEWED JOURNAL AND BOOK PUBLISHING

Journal of Health, Medicine and Nursing
ISSN 2520-4025 (Online)
Vol.4, Issue 5. No.2, pp 12- 22, 2019
www.iprjb.org

THE EFFECT OF AFLATOXIN B1 SERUM ALBUMIN ADDUCTS LEVEL ON SUBJECTS WITH AND WITHOUT LIVER DISEASE IN KITUI AND MAKUENI REGIONS OF LOWER EASTERN KENYA.

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ABSTRACT

Purpose: An outbreak of aflatoxicosis associated with aflatoxin B1contaminated maize grain and flour had been reported over the years in parts of lower eastern Kenya including Kitui and Makueni counties. Ingestion of aflatoxin B1 contaminated food stuff at certain levels cause aflatoxicosis which manifests as hepatoxicity and in severe cases, fulminant liver failure. A study was therefore conducted to evaluate the effect of AFB_1 lysine albumen adducts level and therefore, impact of dietary AFB_1 levels on persons with liver disease.

Methodology: The investigation was conducted as a case-control study where blood samples from appropriately selected subjects was analyzed for exposure and non exposure to dietary aflatoxinB1 (AFB_1). A non probability purposive sampling method was used to choose and divide the study area into strata with 19 clusters. The sample size (n) was determined as 283 for both case and control subjects as per Schelsselman formula (1982). Blood samples were drawn, frozen and stored for analysis and determination of AFB_1 lysine albumen adducts.

Findings: Case subjects had 55.83% (n=158) of serum sample positive for AFB_1 lysine albumin adducts with a level range of 15.50 pg/mg to 135.00 pg/mg and a mean of 42.93 pg/mg (95%; CI: 39.36 to 46.51) $p \leq 0.05$, while the controls with 31.0% (n=88) of positive samples had a lower AFB_1 lysine albumin adducts level range of 3.50 pg/mg to 60.50 pg/mg with a mean of 14.30 pg/mg (95%; CI: 12.23 to 16.36), $p \leq 0.05$. Case subjects had higher mean level for AFB_1 lysine albumin adducts than controls, suggesting higher level of dietary AFB_1 exposure. This study therefore, exposed dietary aflatoxin B1 as one of the endemic etiological agents for liver disease in the region.

Unique contribution to theory, practice and policy: This was the first case – control study in lower eastern Kenya to link serum AFB1 lysine albumin adduct levels to incidences of liver disease. Further, the study shall influence use of innovative methods of preventing infestation of moulds, especially those of the genus *A. flavus* in grains and cereals, which produce aflatoxin B1 and eventually contaminate most types of cereals.

Keywords: *Dietary, aflatoxinB1 lysine albumin adducts, liver disease, Kitui, Makueni, lower eastern Kenya*

1.0 INTRODUCTION

Aflatoxin contamination of food stuff may occur during pre-harvest or post-harvest period, during storage, transportation and processing (Li *et al.*, 2009) and (Rural21, 2013). Continued dietary exposure to aflatoxin B1 (AFB_1), is a major risk factor for hepatocellular carcinoma and general liver damage in populations, particularly in areas where hepatitis B virus (HBV) infection is endemic. Ingestion of higher doses of aflatoxins can result to acute aflatoxicosis which manifests as hepatoxicity or in severe cases fulminate liver failure (Golthardt *et al.*, 2009). To reduce the incidence of aflatoxin poisoning, Codex Allimentarius Commission (CAC), has recommended that the levels of aflatoxins in food stuff for human consumption should be less or equal to 10 ppb (Codex Commission, 2008). Federal Drug Agency (FDA) has regulated that corn or corn products intended for animal feeds should have aflatoxin levels of ≤ 20 ppb (FDA, 2009), while in Kenya, the regulatory threshold of 10 ug/kg of total aflatoxins and 5 ug/kg of AFB_1 in human food is the standard (KEBS,2018a). Aflatoxins also fluoresce strongly in the ultra violet light (ca 365 mm), with B_1 and B_2 producing blue fluorescence, whereas G_1 and G_2 produce green florescence (Vosough *et al.*, 2010). This has been used as a method of categorization of these toxic substances in human food. Once ingested with food, aflatoxin B1 forms AFB_1 lysine albumin adducts classified as carcinogenic and which is a biomarker for aflatoxicosis. Because of the hepatoxicity of aflatoxins, the level of exposure is of particular public health concern. The Food and Agricultural Organization (FAO), and Agro food and Veterinary Diagnostics Organization (AVD), estimates that mycotoxins contaminate 25% of agricultural cereals worldwide (CAST, 1998; AVD, 2013).

The objective of the study was to evaluate the impact of dietary AFB_1 levels among liver disease cases and hence determine whether dietary AFB_1 level in human blood was a causal factor of the disease among the study subjects.

2.0 MATERIALS AND METHODS

2.1 Study design

The study was designed and conducted as a Case - Control study for human subjects which involved collection of blood samples from both cases and controls after which a laboratory serum sample analysis for levels of AFB_1-lysine albumen adducts was conducted. The presence of AFB_1 serum albumin adducts was to determine exposure and non exposure to aflatoxin B1, a major cause of liver damage and a class one carcinogen among the subjects.

The study was conducted in Kitui and Makueni counties including parts of lower Machakos now administratively under Makueni county (Table 1), which are arid and semi arid areas. The study area was stratified into two main strata and nineteen sub clusters (sub strata) based on administrative units (Table 1).

Table 1: Distribution of the sub clusters for both HBV and AFB_1 lysine albumin adducts contaminated serum samples in the strata within the study area.

Kitui County	Makueni County
Kavisuni	Kibwezi
Muthale	Emali
Mutomo	Sultan Hamud
Migwani	Mtito Adei
Kitui	Wote
Nuu	Kathozweni
Tei wa Yesu	Masaku
Mathuki	Makindu
Mwingi	
Kyuso	
Mtito Ndooa	

2.2 Study approvals

The study was granted approvals by Ethical research committee of Kenya medical research institute (KEMRI), through the scientific steering committee (No.2988) and National committee on science, innovation and technology (NACOSTI/P/15/5700/7331), after the vetting procedures for study protocals dealing with human subjects.

2.3 Sampling method

The study employed Non probability stratified purposive sampling method on basis of clinical diagnosis of liver disease among cases and non disease in control subjects. The sample size (n) for the case-control study was determined by use of the Schlesselman (1982), formula as 283 cases and 283 controls (N=566).The subjects were between ages of 12 to 80 yrs for both sexes and had been resident in the area for at least a year.

2.4 Invasive procedure for collection of blood samples

The invasive procedure for collection of blood samples from each case and control subject was undertaken by a qualified lab technologist (phlebotomist) as per the WHO, (2010), guidelines. The phlebotomist used sterile hand gloves, and then assembled equipment including a tourniquet, methanol and 70% alcohol swabs cotton wool for skin disinfection, to be applied over punctured site and red rubber topped 10 ml vacutainer tubes to where 4ml of blood each was collected. Laboratory specimen labels, and forms for recording the case and control sample codes were used in this procedure. The subjects were prepared by also obtaining a verbal consent even if informed consent had been obtained before. Blood was then drawn, transferred to Vacutainer tube, frozen to 10 ^{0}C and transported for analysis.

2.5 Determination of AFB_1 lysine albumin adducts levels

2.5.1 Preparation of samples and AFB_1-albumin standards.

The direct competitive ELISA Kit (Glory® Science Co., Ltd. USA), was used for the total determination of AFB_1-albumin adducts in the human serum samples for both case and controls in the study. The kit manufacturer (Glory Science Co. Ltd USA), had put the kit detection lower limit to 0.3ug/L (0.3ug/mL) with extracts from feed fish, shrimps urine or serum samples.

Each sample serum extract in a $10cm^3$ test tube was diluted using methanol (1:10) solution, then centrifuged for 3 minutes to get the liquid supernant (serum) for the test.

Six aflatoxin-albumin adduct standards, vials each of 1ml, and the concentration 0 ng/ml, 0.1ng/ml, 0.3ng/ml, 0.9ng/ml, 2.7ng/ml, and 8.1ng/ml was arranged in a test tube rack and labelled; S_1, S_2, S_3, S_4 and S_5(Glory Science Co,. Ltd. USA).

2.5.2 Preparation of AFB_1- albumin adduct-enzyme conjugates.

The ELISA kit (Glory® Science Co., Ltd USA), had an already prepared AFB_1-albumin adduct-enzyme conjugate which was used for tests, in both micro-titre and standard wells.

2.5.3 Preparation of TMB-enzyme substrate

The ELISA kit was supplied with an already made enzyme colour marker with TMB-substrate but for accuracy purposes, the solution was prepared by mixing a portion of (1:1), citric acid buffered solution (pH 3.8), containing 325ul of 30% hydrogen peroxide per litre of solution and one portion of a solution of 50.4 mg tetra methyl benzidine (TMB) in an acetone-methanol (1: 9) solution.

2.5.4 Analysis procedure:

Fifty (50) ul, of the standard AFB_1-albumin serum adduct solution was pipetted in duplicate to the pre-coated aflatoxin albumin adduct antibody removable micro-titre plates in the order S_0, S_1, S_2, S_3, S_4 and S_5 representing standard dilutions of 0 ng/ml, 0.3ng/ml, 0.9ng/ml, 2.7ng/ml and 8.1ng/ml. Similarly 50 ul, of sample serum was pipetted into adjacent pre-coated wells. Aliquots of 50 ul, of AFB_1-albumin adducts enzyme conjugate (Glory® Science Co. Ltd USA), was added to all the wells of both the standards and the sample, covered with an aluminium foil and incubated at room temperature (28ºC) for two (2) hours.

The plate was then emptied and washed with saline tween solution (8.55gm sodium chloride dissolved in 1000 ul distilled water, plus 0.25ml of poly oxy ethelene sorbitan monohydrate), and dried by tapping with a blotting paper.

An enzyme substrate (Glory® Science Co. Ltd USA), which consisted of Horse radish peroxidise and tri methyl benzidine, was added and the plates incubated in the dark for 10 minutes, after which the enzyme reaction was stopped by adding 100 ul of 2M sulphuric acid simultaneously into all micro-titre wells. The colour had changed from blue to yellowish.

The intensity of colour in all wells was determined by measuring absorbance at 450nm, using an ELISA reader (Uniskan II® Lab systems, Finland). The absorbance value data for standards and serum samples was entered into computer software (R-ridasoftwin® version 1.60, R-bio pharm., Germany), which used percentage absorbance against known standard aflatoxin adducts

concentrations to draw a standard curve. The software automatically generated AFB_1- albumin adducts levels in parts per billion (ppb) which was converted into pg/mL.

2.5.5 Data analysis

Computer software SPSS version 18.0 was used for analysis of laboratory data on AFB_1 lysine albumin adducts for means ($\bar{X}$), medians, ranges and standard deviations (Sd) and confidence intervals of the means (CI). The confidence interval (CI) statistical manipulations was cross worked with Casio® fx-82EX (Casio, Japan), statistical tool at 95% confidence level and 5% significance level ($0 < p \leq 0.05$).

3.0 FINDINGS AND DISCUSSION

3.1 Case subjects mean AFB1 lysine albumin adducts level in serum samples

The case sample (N= 283) had, 55.83% (n=158) of the subject serum samples positive for AFB_1 lysine albumin adducts with a range of 15.5 pg/mg to 135.0 pg/mg and a mean of 42.93 pg/mg (95%; CI: 39.36 to 46.51) $p \leq 0.05$. Among the centers with higher serum means for AFB_1 lysine albumin adducts, Mathuki health center with 0.706% (n=2) of the positive serum samples, had AFB_1 lysine albumin adducts level range of 68.0 pg/mg to 82.50 pg/mg with a mean of 75.75 pg/mg, while Kathozweni health center with 3.15% (n=9) of subject serum samples had a range of 23.8 pg/mg to 102.80 pg/mg with a mean of 63.80 pg/mg. E mali health center with 1.06% (n=3) of the serum positive sample was in the same category, with a subject serum AFB_1 lysine albumin adducts range of 18.50 pg/mg to 64.80 pg/mg, but with a center adducts mean level of 42.80 pg/mg. For health centers with lower serum mean AFB_1 lysine albumin adducts in this cohort, Mtito ndooa with 1.06% (n=1) of the sample had a range of 18.80 pg/mg to 25.00 pg/mg and an AFB_1 lysine albumin adducts mean of 21.53 pg/mg, while Kyuso health center with 0.35% (n=1) of the positive sample had a mean of 23.80 pg/mg. Tei wa Yesu in this category with 0.71%(n=2) of the sample had an AFB_1 adducts range of 19.80 pg/mg to 31.50 pg/mg and a mean of 25.65pg /mg. Table 2 shows the case subjects AFB_1 lysine albumin adduct levels, range, means and median per health center.

Journal of Health, Medicine and Nursing
ISSN 2520-4025 (Online)
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www.iprjb.org

Table 2: Case AFB_1 lysine albumin adducts level in blood samples per health center

health (centers)	sample (n)	range (pg/mg)	mean (pg/mg)	median (pg/mg)	sd
Mutomo	11	15.80—93.80	34.06	25.80	24.84
Tei wa yesu	2	19.80—31.50	25.65	25.65	5.840
Kitui	9	16.80—47.80	33.28	33.80	9.344
Mtito ndooa	3	18.80—25.00	21.53	20.80	2.583
Mwingi	8	18.50—96.50	44.83	28.65	30.23
Kyuso	1	0.000	23.80	23.80	0.000
Migwani	7	19.80—63.00	38.08	32.00	17.090
Kavisuni	2	19.80—56.50	38.15	38.15	18.350
Nuu	5	15.80—71.80	38.84	32.00	21.030
Muthale	10	19.50—66.50	34.24	27.90	15.930
Mathuki	2	68.00—82.50	75.25	75.75	7.250
Kibwezi	1	0.000	33.80	33.80	0.000
Wote	24	17.50—64.50	34.15	31.25	11.797
Sultan H	7	19.80—74.50	43.66	43.00	21.040
Masaku	52	15.50—97.80	47.38	43.00	22.500
Kathozweni	9	23.80—102.8	62.13	63.80	26.980
Makindu	5	24.00—55.00	40.62	38.80	11.410
Emali	3	18.50—64.80	42.03	42.80	18.910
Mtito Adei	3	53.30—135.0	68.50	68.50	35.417

Note, Sd= standard deviation

Figure 1 show a comparison of case sample mean AFB_1 lysine albumin adducts between health centers, with Mathuki health center having higher subject mean of 75.75 pg/mg, while Mtito Ndooa health center had the lowest AFB_1 lysine albumin mean at 20.80 pg/mg of albumin.

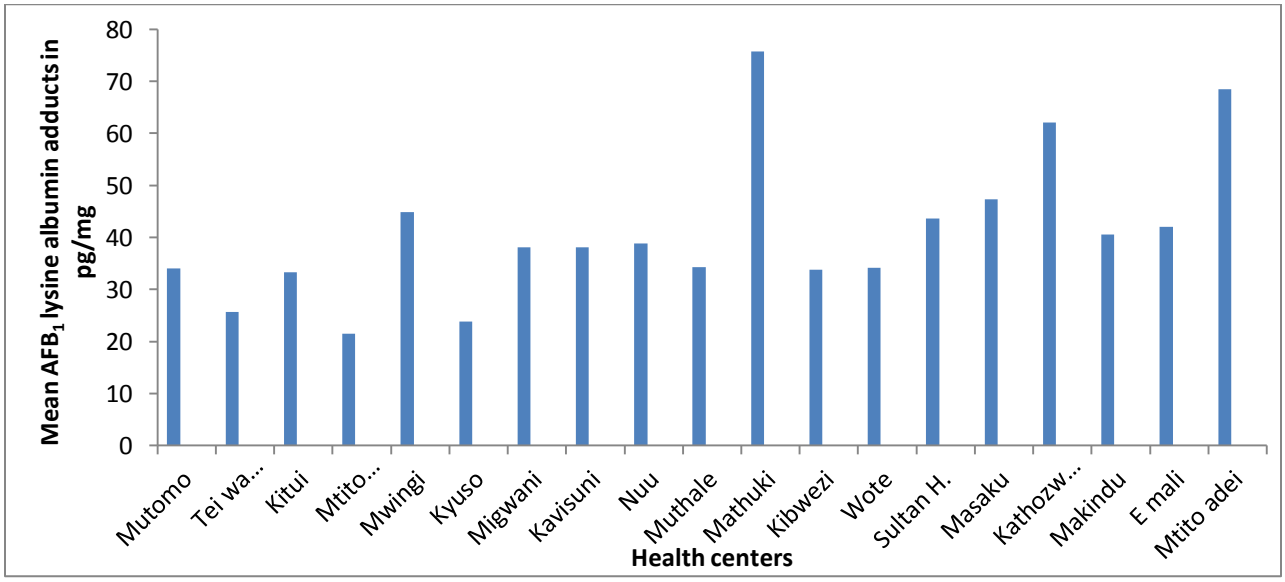

Figure 1: Case mean AFB_1 lysine albumin adducts level in blood samples per center

3.2 Control cohort mean AFB_1 lysine albumin adducts level in serum samples

The control subjects sample (N=283), had 31% (n = 88), of subject serum samples positive for AFB_1 lysine albumin adducts with a level range of 3.5 pg/mg to 60.50 pg/mg and a mean of 14.30 pg/mg (95%; CI: 12.23 to 16.36) $p \leq 0.05$. Within the same cohort, 31.09% (n=88) of subjects had evidence of low key aflatoxicosis as indicated by the quantified mean AFB_1 lysine albumin adduct levels even though they had not been clinically diagnosed with liver disease(Table 3).

Table 3: Control mean AFB_1 lysine albumin adduct level in serum samples per center

health center	positive sample (n)	range (pg/mg)	mean (pg/mg)	median (pg/mg)	sd
Mutomo	8	6.50—12.00	9.03	7.90	2.130
Tei wa yesu	2	5.50—16.50	11	11	5.500
Kitui	7	9.50—29.50	19.73	19.50	6.387
Mtito ndooa	1	0.00	4.50	4.50	0.000
Mwingi	3	5.80—15.60	12.30	15.50	4.596
Kyuso	0	0.00	0.00	0.00	0.000
Migwani	1	0.00	8.40	8.40	0.000
Kavisuni	0	0.00	0.00	0.00	0.000
Nuu	1	0.00	8.00	8.00	0.000
Muthale	5	5.50 —32.50	18.12	18.80	10.452
Mathuki	2	17.00—28.50	22.75	22.75	5.750
Kibwezi	0	0.00	0.00	0.00	0.000
Wote	11	4.50—23.40	12.25	13.00	5.269
Sultan H.	1	0.00	9.50	9.50	0.000
Masaku	33	3.50—40.50	13.90	10.80	9.262
Kathozweni	8	6.40—60.50	23.125	15.70	18.281
Makindu	3	5.80—10.40	8.33	8.80	1.906
E mali	1	0.00	7.50	7.50	0.00
Mtito adei	1	0.00	11.40	11.40	0.00

Note, Sd = standard deviation

Among the control centers with higher mean AFB_1 lysine albumin adducts levels, Kathonzweni health center with 2.83%(n=8) of positive serum samples had a range of 6.4 pg/mg to 60.5 pg/mg and a mean of 23.125 pg/mg, while Mathuki center with 0.71% (n=2) of positive serum sample had a range of 17.00 pg/mg to 28.50 pg/mg with a mean of 22.75pg/mg (Table3). Figure 2 is a comparison chart on mean control subject AFB_1 lysine albumin levels among health centers.

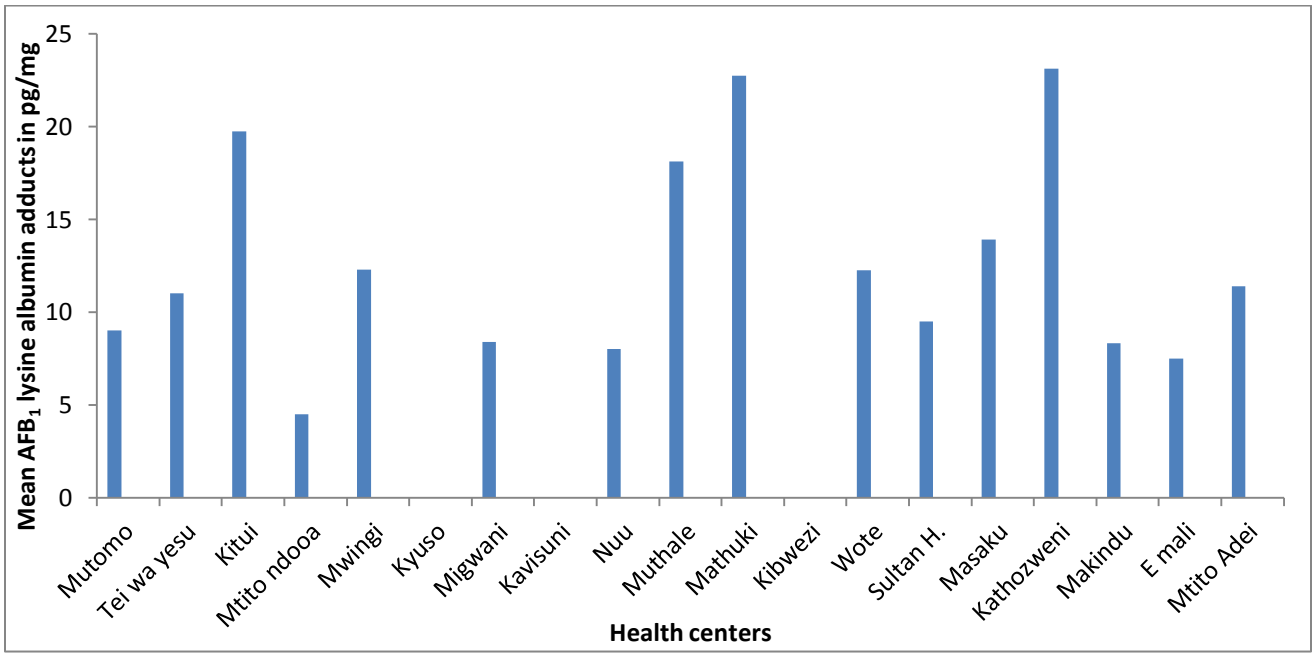

Figure 2: Control subjects mean AFB_1 lysine albumin levels per health center

4.0 SUMMARY, CONCLUSION AND RECOMMEDATION

4.1 Summary

Lower eastern Kenya has had an aflatoxicosis outbreak severally in the near past, with the outbreak of April 2004 generating 317 cases and causing 125 deaths (Lewis *et al.,* 2005). A cross sectional study by the team revealed a high level of AFB_1 contamination of maize grain which was and still his the stable food in this region (Lewis *et al*.,2005). This current case control study evaluated the link between the subject AFB_1 serum albumin adduct mean levels and liver disease (aflatoxicosis) in lower eastern Kenya. Much so because, when mycotoxins are ingested through foodstuffs, aflatoxin B1 through a series of bio chemical reactions is covalently bound to blood proteins specifically albumin to form AFB_1 serum albumin adducts. Aflatoxin B1 is also metabolized primarily in the liver by cytochrome p-450 system, forming the highly reactive AFB_1-8, 9-epoxide which binds to hepatic cell's DNA. This bio chemical reaction forms AFB_1-formalmidopyrimidine DNA adducts which alters normal cell function; hence the macro molecule is highly carcinogenic (Turner *et al.,* 1998). Studies have shown that the half life (1/2) of serum albumin stored under normal temperature is 20 days, hence any chronic exposure to dietary AFB_1 may lead to high circulatory concentration of AFB_1 lysine albumin adducts leading to chronic liver damage, liver disease or sudden toxicities, depending on a patient's immune response in dealing with the toxins (Scholl & Groopman, 2008). This observation agreed with this study were the case subjects were found to have higher mean serum AFB_1 lysine albumin levels at 42.30 pg/mg (95%; CI: 39.36 to 46.51) $p \leq 0.05$, while the control subjects had 14.30 pg/mg (95%; CI: 12.23 to 16.36) $p \leq 0.05$. Since formation of AFB_1 lysine albumin adducts is dose dependent, the accumulation of the albumin adducts correlated well with exposure to

dietary AFB_1 and therefore it was evident that the residents of these regions were highly exposed to dietary AFB_1 (Turner *et al.,* 1998). The lower mean level for AFB_1 lysine albumin adducts in controls who had no evidence of clinical liver disease suggested a certain cumulative threshold for ingested AFB_1 as causal agent for liver disease. This observation agreed with other studies by Muthomi *et al,* (2009) and Muhia *et al,* (2008), which all suggested that, subjects in this region were exposed to higher levels of aflatoxin B1 from dietary maize grain and flour

4.2 Conclusion

This study is significant in that it is the first case - control study in lower eastern Kenya to link the quantified serum levels of AFB_1 lysine albumin adducts to liver disease among the subjects in that region. This evaluation study found the residents of the study area to be highly exposed to dietary AFB_1 as determined by the quantified levels of AFB_1 lysine albumin adducts in case and control subjects. Further, since some controls were found to have certain low levels of AFB1 lysine albumin adducts, it follows that a certain threshold (level) of aflatoxin B1 in serum was required for clinical manifestation of liver toxicity due to dietary AFB1 exposure. This study however exposed dietary AFB_1 as an endemic etiological agent for liver disease in the region.

4.3 Recommendation

This study recommends a public health campaign to educate the residents of lower eastern Kenya on proper drying and storage of grain and grain products including maize flour. Further, mass clinical screening for liver conditions could be instituted in the region for early treatment of liver diseases including those whose etiological agents are not related to aflatoxin B1.

ACKNOWLEDGEMENTS

This study was carried out in lower eastern Kenya in nineteen (19) health centers which also included major county referral health facilities where serum samples were collected. Part of the work was guided in Kenya medical research institute (KEMRI), and Bora biotech laboratories, Kabete. Laboratory analysis of serum samples was supervised by Prof. J. Gathumbi. The principal investigator, collected the samples, did laboratory testing under supervision and statistically analyzed the data. This work is part of certification process work for Pius k Mutisya. We thank the study subjects/participants for granting this study the serum samples used. This work is published with permission of the Director, KEMRI and the approval of the JKUAT appointed supervisors Yeri Kombe, Fred Wamunyokoli, Charles F. L. Mbakaya and Joseph Gathumbi who are co authors of this paper.

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