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Assessment of aflatoxin M_1 and enteropathogenic microorganism levels in milk samples vended in Cross River State, Nigeria

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Abstract: Aflatoxin M_1 and enteropathogenic microorganism levels in milk samples vended in Cross River State were investigated. Thirty one milk samples were purchased from supermarkets and markets across the three (3) senatorial districts of Cross River State during August to November 2012. The milk samples were grouped into three (3) categories; A (Evaporated milk samples), B (Powdered milk samples), C (Infant milk formula). The milk samples were analyzed for aflatoxin M_1 (AFM_1) by competitive enzyme linked immunosorbent assay (ELISA) while enteropathogens (Salmonella, Escherichia coli, Mould, Mesophilic Aerobic bacteria and Coliform) were cultured for microbiological sensitivity test using standard methods. Aflatoxin M_1 (AFM_1) was found in 100 percent of all the milk samples that were analyzed in this study. The contamination levels ranged from 0.06µg/l to 0.07µg/l, while the mean value was 0.07µg/l. There were no significant differences ($P>0.05$) between the mean concentrations of AFM_1 of the milk samples among the different categories. All the different milk samples (100%) exceeded the European Union maximum acceptable levels (0.05µg/l). None of the milk samples exceeded the Nigerian permissible limit (0.5µg/l). Salmonella, Escherichia Coli and Coliform bacteria were not detected in the milk samples. Aerobic Mesophilic bacteria and Mould were present in the milk samples but did not exceed the standard of 10^5cfu/ml for aerobic mesophilic bacteria and 10^2cfu/ml for mould. It is concluded that the milk samples vended in Cross River State contain aflatoxin M_1 and detectable enteropathogen levels which fall within Nigerian regulatory limits.

Keywords: Aflatoxin M_1, Pasteurized Milk, Measurement, Enteropathogens, Elisa, Nigeria

1. Introduction

Aflatoxins are a group of closely related heterocyclic compounds produced predominantly by two filamentous fungi, *Aspergillus flavus* and *Aspergillus parasiticus*. *Aspergillus flavus* produces only B aflatoxins (AFB_1 + AFB_2), while *Aspergillus parasiticus* produces both B and G aflatoxins (AFG_1 + AFG_2) (Gurbay *et al*., 2006). Aflatoxin M_1 is the hydroxylated metabolite of aflatoxin B_1. Aflatoxin M_1 (AFM_1) is found in infant milk formula (FDA, 2007) as well as in milk products, including non-fat dry milk, cheese, and yogurt (Tajkarimi, 2007).

Aflatoxin is the most potent natural carcinogenic substance and has been linked with higher prevalence of hepatocellular cancer in Africa (Strosnider *et al*., 2006). International Agency for research on cancer (IARC, 1993) classified AFM_1 as class 2B human carcinogen. There is a high risk of Hepatitis B and Hepatitis C carriers developing liver cancer when they are exposed to aflatoxin (Williams *et al*., 2004). Exposure of infants to AFM_1 is worrisome, because they are considered more susceptible to its adverse effects, and their capacity for biotransformation of carcinogens is generally slower (Lopez, 2003).The toxicity activity of aflatoxin M_1 (AFM_1) is due to their capacity to interact with nucleic acids, nucleoproteins and protein synthesis (Dewick, 2009).

The biological value of milk is second to eggs in regards to essential amino acids, energy, calcium and vitamins (Anderson *et al*., 1999). Milk is an important source of protein, minerals, vitamins and fats in human diet which approximately comprises of 87% water, 3.7% protein, 4.9% lactose, 0.7% ash and 3.6% fat (Ramesh *et al*., 2008). This complex biochemical composition, render milk an excellent

medium for both pathogenic and spoilage microorganisms (Okonkwo, 2011). Outbreaks of milk-borne diseases have occurred despite pasteurization, as a result of either improper pasteurization or product recontamination (Nebedum and Obiakor, 2007).

Dairy products are consumed by millions on daily basis worldwide and as such the potential of food-borne illness is a major concern to producers, regulators, and consumers (Bryne, 2004). Most of the food-borne illnesses associated with milk consumption are linked to post-pasteurization contamination (Olsen *et al*., 2004). Post-pasteurization contamination of milk is mostly by contaminated hands of dairy workers, unsanitary utensils and polluted water supply (Pantoja *et al*., 2009). Detection of specific pathogens (*E.coli*, coliform, mould) and their toxins are used as index of contamination of milk and its products with possibility of presence of pathogens which may constitute health hazards to consumers (Parekh and Subhash, 2008).

Many countries have carried out studies about the incidence of aflatoxin M_1 and enteropathogenic quality in milk samples. In most of them, samples have been found to exceed the limit imposed by many countries of 0.5 µg/l (Karim *et al*., 1998). Also, most of them revealed high level of contamination of Aerobic mesophilic bacteria, coliform, *Salmonella*, *Escherichia coli*, with counts exceeding the recommended acceptable levels (Aboloma, 2008). Regulatory limits throughout the world are highly variable, depending on the degree of development and economic involvement of countries, and may vary from one country to another (Van Egmond, 1991). The European Community prescribes that the maximum level of AFM_1 in liquid milk and dried or processed milk products should not exceed 50 ng/kg = 50ppt (0.05 µg/l) (FAO, 2001). However, according to Nigeria and US regulations the level of AFM_1 in milk should not be higher than 500 ng/kg = 500ppt (0.5 µg/l (FAO, 2003). In Nigeria, there are very few data about aflatoxin M_1 incidence in milk samples. The potential hazard of aflatoxins to human health especially infants, who are very vulnerable to diseases, have led to worldwide monitoring programs for the toxin in various commodities. Hence, there is a need for the evaluation of milk samples currently available in Cross River State and compare the results with maximum tolerable limits in milk that is accepted by the European Union and Nigeria.

2. Materials and Method

2.1. Sample Collection

Thirty one (31) milk samples were collected from supermarkets, and markets, across the three senatorial districts of Cross River State. The 31 samples were drawn from different brands which are a representation of milk products marketed in Cross River State. All samples were transported and kept hermetically sealed after purchase until the day of analysis. Ten (10) different milk samples (brands) which included four (4) evaporated milk, two (2) powdered milk , and four (4) infant formula was purchased from Northern senatorial district. Also, Eight (8) different milk samples (brands) which included two (2) evaporated milk, four (4) powdered milk, and two (2) infant formula was purchased from Central senatorial district while thirteen (13) different milk samples (brands) which comprises of five (5) evaporated milk, four (4) powdered milk, and four (4) infant formula was purchased from Southern senatorial district.

The milk samples were categorized into three (3):

- Category a (11 samples) – evaporated milk.
- Category b (10 samples) – powdered milk.
- Category c (10 samples) – powdered infant formulae.

2.2. Samples Preparation

2.2.1. Evaporated Milk

For evaporated milk, 20ml of milk was chilled to 10ºC and then centrifuged (Uniscope Centrifuge with model no.112) for 10minutes at 3500 g. The fatty layer was removed and 100 microlitre of the defatted milk or supernatant was applied directly in the ELISA microtiter plate.

2.2.2. Milk Powder

Ten grams of powdered milk was placed in a flask, and 100ml of distilled water was added. The mixture was stirred for 5minutes and then centrifuged (Uniscope Centrifuge with model no.112) at 3500 g for 10minutes at 10^{O}C temperature. After centrifugation, the upper fatty layer was removed and 100 microlitre of the skimmed milk (defatted supernatant) was used for ELISA analysis.

2.2.3. ELISA Test Procedure

The presence of AFM_1 in the milk samples was detected with ELISA (Ridascreen®Aflatoxin M_1 (Art No.: R1121, R-Biopharm GmbH, Germany) as described by Gurbay *et al*., 2006.

Ninety six (96) microtiter wells were inserted into the microwell holder for all standards and samples. One hundred microliters (100µl) of standard solution and prepared samples (100µl) was added in separate wells and incubated for 60minutes at room temperature (22-25^{O}C) in the dark. The liquid was then poured out and the microwell holder was tapped upside down vigorously (three times in a row) against absorbent paper to ensure complete removal of the liquid from the wells. Then, the wells were washed twice with 250µl of distilled water. One hundred microliters (100µl) of the diluted enzyme conjugate (peroxidase conjugated AFM_1) were added and incubated for 60minutes at room temperature (25^{O}C) in the dark. The wells were again washed with 250µl of distilled water as described above. In the next stage, 100µl of substrate/chromogen was added to each well and mixed thoroughly and incubated for 30minutes at room temperature in the dark. Then 100µl of the stop solution (1N H_2SO_4) was added to each well and mixed, and the

absorbance was measured at 450 nm in an ELISA reader (Stat Fax 303 with serial no.30311451).

2.2.4. Enteropathogenic Assay

The enteropathogenic microorganisms were evaluated using standard microbiological procedures. Twenty five grams (25g) of milk samples were mixed with 225ml of Buffered peptone water (BPW) for at least 1 minute. Decimal dilutions of the homogenized samples were prepared in 9 ml of BPW and plated in duplicate onto specific media.

The milk samples were examined for Total Aerobic Mesophilic bacteria, *Escherichia coli*, *Salmonella*, Coliform, and Mould. All selective media were prepared according to standard procedure. Enumeration of Total Aerobic Mesophilic bacteria was performed on Plate Count Agar (PCA) incubated at 35ºC for 72hrs. The count of *Escherichia coli* was performed on Violet Red Bile Agar (VRBA) incubated at 37ºC for 48hrs. Enumeration of Mould was performed on Potato Dextrose Agar (PDA) incubated at 25ºC for 7 days. Enumeration of *Salmonella* was performed on *Salmonella Shigella* Agar (SSA) incubated at 37ºC for 48hrs. Enumeration of Coliform was performed on Lauryl Sulphate Tryptose Broth (LSTB) incubated at 37ºC for 48hrs.

2.3. Statistical Analysis

The data obtained were analyzed using SPSS (Statistical Package for Social Sciences) 10.0 for windows. Probability less than 0.05 was considered significant. Toxin concentrations in the samples were compared with standard concentration. Chi-square test was also used to test for independence.

3. Results

In this study, a total of 31 pasteurized milk samples were analyzed for aflatoxin M_1 and enteropathogens by competitive ELISA technique and microbiological sensitivity test using standard methods. Table 1, shows the concentrations of Aflatoxin M_1 in the milk categories. The prevalence rate of AFM_1 contamination in the various milk samples was 100%. In other words, all the milk samples (100%) were contaminated with values ranged from 0.06 to 0.07 µg/l. In category A, eleven (11) evaporated milk samples (100%) were contaminated with levels ranging from 51 to 70 ppt. In category B, ten (10) powdered milk samples (100%) were contaminated with levels ranging from 51 to 70 ppt. In category C, ten (10) infant milk formulae (100%) were contaminated with AFM_1 levels ranging from 51 to 70 ppt. In total, 100% of AFM_1-contaminated milk samples exceeded the European Union Regulation (50 ppt) (0.05µg/l). However, none of the various milk samples exceeded the limit (500 ppt) (0.5 µg/l) set by National Agency for Food and Drug Administration Control (NAFDAC) for Aflatoxin M_1. There was no significant difference (P>0.05) among the milk categories.

Table 2, shows the enteropathogenic status of the milk samples. Category B had the highest bacteria count of 195 cfu/ml. However, the level did not exceed the standard of 10^5cfu/ml set by the Food and Agricultural Organization (FAO). Category C had the highest mould count of 26 cfu/ml when compared to other categories. However, the level did not exceed the standard limit for mould (10^2cfu/ml). *Escherichia Coli,* Coliform*, Salmonella* were all absent or not detected in all the milk samples.

Table 1. *Aflatoxin M_1 Concentration in the various Milk Categories*

Milk sample category	Samples tested (n)	Proportion of percentage (%)	Number of percent of samples with AFM1 in ppt (ng/l) ranges (AFM1 standard ranges)						Proportion of sample exceeding Eu legal limit > 50ppt (0.05µg/l)	Proportion of samples exceeding U.S/Nigeria legal limit>500ppt (0.5µg/l)	Quantity of AFM1 (µg/l)			
			ND	<20	21-30	31 – 50	51 – 70	71 – 90			mean±SEM	SD	Min	Max
Category A	11	35.48	0(0)	0(0)	0(0)	0(0)	11(100)	0(0)	11(100)	0(0)	0.07±0.001	0.005	0.06	0.07
Category B	10	32.26	0(0)	0(0)	0(0)	0(0)	10(100)	0(0)	10(100)	0(0)	007±0.000	0.000	0.07	0.07
Category C	10	32.26	0(0)	0(0)	0(0)	0(0)	10(100)	0(0)	10(100)	0(0)	0.07±0.001	0.004	0.06	0.07
TOTAL	31	100							31(100)	0(0)	0.07±0.00	0.003	0.06	0.07

No significant difference (P>0.05) among the categories.
EU: European Union;
ND: Not detected;
SEM: Stand Error Mean;
Category A:Evaporated milk samples
Category B:Powdered milk samples
Category C:Infant milk formulae

Table 2. *Enteropathogenic status in the various milk categories*

Samples category (n)	Enteropathogenic-organisms	No. of counts (Cfu/ml)	Food and Agriculture Organization/ Nigeria legal limit	No. of samples Exceeding FAO/NAFDAC Limit
	Escherichia coli	-	0	Null
	Salmonella	-	0	Null
Category A(11)	Mould	2	10^2	Null
	Coliform bacteria	-	<5	Null
	Aerobic mesophilic bacteria	2	10^2	Null
	Escherichia coli	-	0	Null
	Salmonella	-	0	Null
Category B(10)	Mould	-	10^2	Null
	Coliform bacteria	2	10^2	Null
	Aerobic mesophilic bacteria	195	10^5	Null
	Escherichia coli	-	0	Null
	Salmonella	-	0	Null
Category C (10)	Mould	26	10^2	Null
	Coliform bacteria	-	0(or <3)	Null
	Aerobic mesophilic bacteria	2	10^3	Null

No significant difference (P>0.05) among the categories
Key:
FAO – Food and Agriculture Organization;
NAFDAC – National Agency for Food and Drug Administration and Control;
CFU/ML – Colony Forming Unit Per Milliliter.

4. Discussion

The occurrence of aflatoxin M_1 in milk is a serious global health problem, particularly in developing countries and many countries have set threshold limit for milk used by adults and infants. In Nigeria, despite a considerable progress in food industry, there is little or no data available on contamination levels of milk and other dairy products with aflatoxin M_1. In this study, aflatoxin M_1 levels, and enteropathogenic microorganism levels were assessed in commonly consumed milk commodities vended in Cross River State, Nigeria. Aflatoxin M_1 (AFM_1) was detected in all the milk samples (100%) used in this study. All the milk samples had concentrations of aflatoxin M_1 above the threshold (0.05µg/kg) set by European Commission. However, all the milk samples were generally below the allowable limit (0.5µg/kg) specified by the National Agency for Food and Drug Administration and Control (NAFDAC), in Nigeria. This observation agrees with the findings from other studies (Celik *et al*., 2005; Roussi *et al*., 2002; Rastogi *et al*., 2004). Levels of aflatoxin M_1 above the levels found in the present study have also been reported in some milk samples (Sefidgar *et al*., 2011). The levels of aflatoxin M_1 in this study may be attributed to high relative humidity, temperature, storage duration which is characteristics of the tropical and sub-tropical regions of the world where Nigeria is located. There was no significant difference (P>0.05) among the milk categories. The implication of this research is that, most of the consumers in Cross River State would have been consuming aflatoxins. Though, in relatively small amount, but, prolong intake of these aflatoxins may constitute a health hazard. Therefore, infant milk, liquid or evaporated milk, and powdered milk for infants, children, adults and the aged must be routinely tested for aflatoxin M_1 presence at every step of manufacturing and marketing.

The quality of milk is determined by aspects of composition and hygiene. In this study thirty one (31) milk samples were tested for Salmonella, Escherichia Coli, Coliform, Mould, and Total aerobic mesophilic bacteria. The results from this study were compared against the microbiological requirements standard (standard 1.6.1) of the Australian New Zealand food standards code and the National Agency for Food and Drug Administration and Control (NAFDAC). A high bacteria count reduces the shelf life of milk and enhances the risk of milk-borne infections and intoxication (Aboloma, 2008).

Coliform Bacteria:

Coliforms in general are indicators of faecal contamination. Coliform bacteria are usually used as marker organisms in the examination of pasteurized milk and ice cream. According to regulations of the European Union (Directive 92/46/EEC), coliform bacteria in pasteurized milk should not exceed 5cfu/ml. In this study, none of the milk samples tested positive for coliform bacteria. This zero level contamination rate may be due to very good hygienic conditions during milking processes, handling and transportation of the milk and the way it is offered for sale.

Escherichia coli:

Escherichia coli strains are a common part of the normal facultative anaerobic microflora of the intestinal tracts of humans and warm-blooded animals. Presently, four (4) main types of pathogenic *E.Coli* have been associated with food-borne disease: enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC) and enterohaemorrhagic *E.coli* (*E.coli* 0157:H7; EHEC). Enterotoxigenic (ETEC) are a major cause of infantile diarrheoa in less developed countries, suggesting that children during the first 2-3 years of life may suffer from as many as two to three clinical infections with ETEC per child per year (Blach *et al*., 1982).

In this study, no attempt was made to differentiate strains of isolated *E.coli*. A high contamination rate of *E.coli* in general does not necessarily implicate public health risk, but is an indicator for potential risk. In the samples analyzed, no samples were positive for Escherichia coli.

Salmonella spp:

In this study, none of the samples contained salmonella spp. Salmonellae is sensitive to heat treatment and is readily destroyed at milk pasteurization temperatures.

Aerobic Mesophilic Bacteria:

The results in this study as shown in Table 2, showed that category B had the highest bacteria count when compared to category A and C. However, the level did not exceed the standard of 10^5cfu/ml According to the Food and Agricultural Organization (FAO, 1992) and World Health Organization (WHO, 2003); standard limit for aerobic mesophilic bacteria count should be less than 10^5cfu/ml. The high bacteria count in this study might be attributed to factors such as the environment, which include exposure of the milk to air, post production operation and personal hygiene of the milk handlers (Aboloma, 2008).

Moulds:

Milk samples were analyzed for the presence of moulds. This micro-organism is very common as a source for spoilage in milk products. As shown above (Table 2), Category C (Infant Milk Formula) showed the highest count of mould (26 cfu/ml). However, it did not exceed the standard limit for mould (10^2cfu/ml). The mould count might be due to both its initial level during manufacturing and poor storage conditions, leading to growth of the mould in the product.

5. Conclusion

The results obtained in this study indicate that milk samples vended in Cross River State contain aflatoxin M_1 and detectable levels of enteropathogens. As milk and milk products are important sources of calcium and are generally popular dietary choices of both mother and child, the contamination of these products by a carcinogenic toxin, such as aflatoxin M_1 during the early vulnerable stages of development is concerning. Therefore, milk and milk products have to be inspected and controlled continuously for aflatoxin and enteropathogenic contamination. Monitoring aflatoxin and enteropathogen levels should be part of quality control procedures in dairy factories, particularly the ones providing infant's milk. Finally, it is concluded that milk samples marketed in Cross River State contain aflatoxin M_1 and detectable enteropathogen levels which fall within Nigerian regulatory limit

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