Assessment of aflatoxin M₁ and enteropathogenic microorganism levels in milk samples vended in Cross River State, Nigeria

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Abstract: Aflatoxin M₁ 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 M₁ (AFM₁) 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 M₁ (AFM₁) 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 AFM₁ 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 10⁵ cfu/ml for aerobic mesophilic bacteria and 10² cfu/ml for mould. It is concluded that the milk samples vended in Cross River State contain aflatoxin M₁ and detectable enteropathogen levels which fall within Nigerian regulatory limits.

Keywords: Aflatoxin M₁, 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 (AFB₁ + AFB₂), while Aspergillus parasiticus produces both B and G aflatoxins (AFG₁ + AFG₂) (Gurbay et al., 2006). Aflatoxin M₁ is the hydroxylated metabolite of aflatoxin B₁. Aflatoxin M₁ (AFM₁) 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 AFM₁ 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 AFM₁ 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 M₁ (AFM₁) 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 M1 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 AFM1 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 AFM1 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 M1 incidence in milk samples. The potential hazard of aflatoxins to human 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 10°C and then centrifuged (Uniscope Centrifuge with model no.112) for 10minutes at 3500 g. The fat layer was removed and 100 microlitre of the defatted milk or supernant 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°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 AFM1 in the milk samples was detected with ELISA (Ridascreen®Aflatoxin M1, (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°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 AFM1) were added and incubated for 60minutes at room temperature (25°C) in the dark. The wells were again washed with 250µl of distilled water as described above. In the next stage, 100µl of the stop solution (1N H2SO4) 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 35°C for 72hrs. The count of Escherichia coli was performed on Violet Red Bile Agar (VRBA) incubated at 37°C for 48hrs. Enumeration of Mould was performed on Potato Dextrose Agar (PDA) incubated at 35°C for 72hrs. The count of Salmonella Shigella agar (SSA) incubated at 37°C for 48hrs. Enumeration of Coliform was performed on Lauryl Sulphate Tryptose Broth (LSTB) incubated at 37°C 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 concentrations. 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 M₁ and enteropathogens by competitive ELISA technique and microbiological sensitivity test using standard methods. Table 1, shows the concentrations of Aflatoxin M₁ in the milk categories. The prevalence rate of AFM₁ 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 AFM₁ levels ranging from 51 to 70 ppt. In total, 100% of AFM₁-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 M₁. 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 10⁵ cfu/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 (10⁴ cfu/ml). Escherichia Coli, Coliform, Salmonella were all absent or not detected in all the milk samples.
but, prolong intake of these aflatoxins may constitute a
progress in food industry, there is little or no data available
No significant difference (P>0.05) among the categories
NAFDAC – National Agency for Food and Drug Administration and Control;
the levels found in the present study have also been reported in some milk samples (Sefidgar
River State, Nigeria. Aflatoxin M
commonly consumed milk commodities vended in Cross
enteropathogenic microorganism levels were assessed in
levels of aflatoxin M
allowable limit (0.5µg/kg) specified by the National
Agency for Food and Drug Administration and Control
findings from other studies (Celik
2002; Rastogi
et al., 2005; Roussi
et al., 1982).).

4. Discussion

The occurrence of aflatoxin M₁ 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 M₁. In this study, aflatoxin M₁ levels, and enteropathogenic microorganism levels were assessed in commonly consumed milk commodities vended in Cross River State, Nigeria. Aflatoxin M₁ (AFM₁) was detected in all the milk samples (100%) used in this study. All the milk samples had concentrations of aflatoxin M₁ 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 M₁ above the levels found in the present study have also been reported in some milk samples (Sefidgar et al., 2011). The levels of aflatoxin M₁ 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 M₁ 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 diarrhoea 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).

Table 2. Enteropathogenic status in the various milk categories

<table>
<thead>
<tr>
<th>Samples category (n)</th>
<th>Enteropathogenic-organisms</th>
<th>No. of counts (CFU/ml)</th>
<th>Food and Agriculture Organization/ Nigeria legal limit</th>
<th>No. of samples Exceeding FAO/NAFDAC Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Category A(11)</td>
<td>Escherichia coli</td>
<td>-</td>
<td>0</td>
<td>Null</td>
</tr>
<tr>
<td></td>
<td>Salmonella</td>
<td>-</td>
<td>0</td>
<td>Null</td>
</tr>
<tr>
<td></td>
<td>Mould</td>
<td>2</td>
<td>10²</td>
<td>Null</td>
</tr>
<tr>
<td></td>
<td>Coliform bacteria</td>
<td>2</td>
<td>&lt;5</td>
<td>Null</td>
</tr>
<tr>
<td></td>
<td>Aerobic mesophilic bacteria</td>
<td>2</td>
<td>10²</td>
<td>Null</td>
</tr>
<tr>
<td></td>
<td>Escherichia coli</td>
<td>-</td>
<td>0</td>
<td>Null</td>
</tr>
<tr>
<td></td>
<td>Salmonella</td>
<td>-</td>
<td>0</td>
<td>Null</td>
</tr>
<tr>
<td>Category B(10)</td>
<td>Mould</td>
<td>-</td>
<td>10²</td>
<td>Null</td>
</tr>
<tr>
<td></td>
<td>Coliform bacteria</td>
<td>2</td>
<td>10²</td>
<td>Null</td>
</tr>
<tr>
<td></td>
<td>Aerobic mesophilic bacteria</td>
<td>195</td>
<td>10³</td>
<td>Null</td>
</tr>
<tr>
<td></td>
<td>Escherichia coli</td>
<td>-</td>
<td>0</td>
<td>Null</td>
</tr>
<tr>
<td></td>
<td>Salmonella</td>
<td>-</td>
<td>0</td>
<td>Null</td>
</tr>
<tr>
<td>Category C(10)</td>
<td>Mould</td>
<td>26</td>
<td>10²</td>
<td>Null</td>
</tr>
<tr>
<td></td>
<td>Coliform bacteria</td>
<td>-</td>
<td>0(or &lt;3)</td>
<td>Null</td>
</tr>
<tr>
<td></td>
<td>Aerobic mesophilic bacteria</td>
<td>2</td>
<td>10³</td>
<td>Null</td>
</tr>
</tbody>
</table>

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 Millilitier.
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. Salmonella 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 10^5 cfu/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 10^5 cfu/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 (10^5 cfu/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 M1 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 M1 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 M1 and detectable enteropathogen levels which fall within Nigerian regulatory limit.

References


