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OCCURRENCE AND LEVEL OF AFLATOXIN M1 IN FRESH RAW COW MILK WITHIN ZARIA METROPOLIS

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ABSTRACT
Food safety is one of the major problems currently facing the world. According to the Food and Agriculture Organization of the United Nations (FAO), a quarter of the world's food crop is spoiled by filamentous fungi and thus should be rejected for food safety reasons. Aflatoxins are the most widely known and distributed mycotoxins in foods and feeds. They are potent carcinogenic, teratogenic and mutagenic agents. Dairy cattle when fed with feed contaminated with Aflatoxin B1 may excrete Aflatoxin M1 in milk as consequences of dietary exposure. The aim of this research work was to determine the occurrence and levels of AFM1 in fresh raw cow milk samples. Fifteen (15) samples were collected from five (5) different sampling sites within Zaria metropolis namely; Dan-Magaji, Kufena, Gabari, Hanwa and Tudun-wada, three (3) samples each from the sampling sites. The samples were screened for AFM1 contamination using a rapid test strip specific for milk samples (Ring Biotechnology Co., Ltd. Art no.:100004-96T). AFM1 contaminated samples were subjected to HPLC analysis to determine the extent of contamination. The result was analysed using 'ANOVA' single factor and Duncan's multiple range test was used to separate the means. All the samples collected were contaminated with AFM1 above the European Union set limit ≤ 0.05µg/L. Samples from Dan-magaji had the highest level of contamination with an average of 101.02µg/L, followed by Kufena, 62.96µg/L, Gabari, 60.71µg/L, Tudun-wada, 36.95µg/L while Hanwa had the least contamination 31.61µg/L. The mean AFM1 in Dan-Magaji was significantly different from means in other locations and this may be due to differences in type of feed given to the animals, degree of AFB1 contamination in the feed and metabolic activities of the lactating animal. Regulatory agencies should adhere to strict monitoring to ensure that AFM1 level did not exceed the acceptable limit in milk and milk products.
Key word; Aflatoxin B1, Aflatoxin M1, Milk, Pasteurization, Regulatory agencies.

INTRODUCTION

Food safety is one of the major problems currently facing the world; accordingly, a variety of studies have been conducted to discuss methods of addressing consumer concerns with various aspects of food safety (Nielsen *et al.,* 2009). According to the Food and Agriculture Organization of the United Nations (FAO and WHO, 2017), a quarter of the world's food crop is spoiled by filamentous fungi and thus should be rejected for food safety reasons at the expense of the food supply of a steadily rising world population. More than 250 mold types that produce mycotoxins are particularly problematic. Among the approximately 300 known mycotoxins, aflatoxins are the most important (Hans *et al.,* 2016).

Aflatoxin M1 (AFM1) is the most rapidly formed metabolite of AFB1 produced by the liver in cattle following ingestion of the parental toxin in contaminated feed (Patterson *et al.,* 2008). Similar to other aflatoxins, AFM1 has been classified in Group 1 as carcinogenic to humans since sufficient evidence exists for its hepato-carcinogenicity in humans (International Agency for Research on Cancer, 2002). Approximately 0.5-5% of AFB1 is transferred in milk as AFM1. After ingestion of cattle feed contaminated with AFB1. Studies have shown that AFM1 could quickly appear (within 12 hour) after ingestion of AFB1 by lactating cows and its concentration decreased gradually to under the limit of detection within 72 hours after removal of AFB1 contaminated feeds (Fallah, 2010).

Animal milk is of great importance in human nutrition, due to its rich nutrient content and beneficial health effects (Akinyemi *et al.,* 2021). It is a good source of macro- and micronutrients. Milk is affordable and can help diversify diets in developing countries (Mc-Mahon, 2013). It is widely consumed either as raw/unprocessed or processed (condensed, pasteurized, powdered, liquid, heat-treated or UHT-treated) milk (Becker *et al.,* 2016).

In Nigeria, average per capita milk consumption is estimated at 10-20 L per year (Nigerian Dairy Development Program, 2018). Cow milk is the most widely consumed either in raw form or as locally fermented products e.g. yogurt (*fura da nono*) and cheese which serve as complementary meals consumed typically at least once a day by all age groups in the rural parts of northern Nigeria (Adewumi *et al.,* 2015). The dairy sector play a very important role in Nigerian agriculture contributing about 23.78% of the agricultural Gross Domestic Product (National Bureau of Statistics, 2021).

With increased production and consumption of milk and dairy products, there is concern about the presence of hazards in milk and their effect on human health. One such hazard is aflatoxin (AF) (Mc-Mahon, 2013). The rate of AFM1 excretion in milk depends on different nutritional and physiological factors, such as feeding regimen, ingestion and digestion rates, animal health, hepatic biotransformation capacity, and lactation period (Duarte *et al.,* 2013). Furthermore, AFM1 is heat stable in raw processed milk and dairy products and is not completely destroyed by pasteurization, sterilization, and other food processing procedures (Oruc *et al.,* 2006).

Several effects of aflatoxin exposure are well studied. Acute aflatoxicosis in humans and animals is reported worldwide. Aflatoxicosis due to chronic exposure at high and moderate concentrations can lead to acute primary Aflatoxicosis. Symptoms include hemorrhage, acute liver damage, edema, digestion problems and death (Peromingo *et al.,* 2016). Aflatoxins are also mutagenic and carcinogenic. Mutagenic effect leads to mutation in genetic code, alteration in DNA which leads to chromosomal breakage, rearrangements, loss or gain of chromosome or changes within a gene (Malik *et al*., 2014).

The European Union (EU) has set the limit of aflatoxin B1 (AFB1) in feed for dairy cattle to be 5 ng/kg, while the limit for aflatoxin M1 (AFM1) in milk is 50 ng/kg which was adopted in Nigeria by the National Agency for Food and Drug Administration and Control (NAFDAC) as 50 ng/L and 25 ng/L in milk intended as food for adults and infants, respectively. The Codex Alimentarius limit is 500 ng/kg (European Commission, 2002).

The main objective of this research is to screen samples for AFM1 contamination and quantify the level of AFM1 using HPLC analysis.

MATERIALS AND METHODS

The study was conducted in Zaria local government of Kaduna State, One (1) litre each of fifteen (15) fresh raw cow milk samples were collected and labelled from five (5) different Fulani settlements namely; Dan-magaji, Gabari, Kufena, Hanwa and Tudun-wada within Zaria metropolis. The samples were transported in ice packs to Multiuser Science Research Laboratory ABU Zaria for analysis.

Rapid test kit specific for milk samples was used to screen samples based on manufacturer's instructions (Ring Biotechnology Co., Ltd. Art no.:100004-96T). The kit utilizes high affinity of monoclonal antibody against AFM1 which can easily identify its contamination in milk. Its AFMI detection limit can meet both EU and USA acceptable limits.

Quantification of aflatoxin M1 level from positive samples using HPLC analysis

Aflatoxin M1 standard was obtained from R-Biopharm (Darmstadt, Germany). HPLC grade methanol, acetonitrile and water were obtained from Fisher Scientific Company, UK. The liquid chromatographic system (1260 Infinity Agilent Technologies, USA) consisted of a HPLC pump, an auto injector, a column oven, and a fluorescence detector. The HPLC conditions for analysis of AFM1 were as follows: column, Hypersil 5AA-ODS 200 x 2.1mm (Agilent Technologies, USA); column temperature, 25°C; mobile phase, water: acetonitrile: methanol (60:30:10); flow rate, 0.7ml/min, retention time 2m, injection volume 5µl and detector, fluorescence spectrophotometer (excitation 360 nm; emission 440 nm).

Extraction and purification of milk samples

The extraction procedure was performed as previously described by Ruangwises and Ruangwises (2013). One hundred (100) ml of raw cow milk sample was measured into a 100ml glass beaker and were placed in a freezer to attain a temperature of 4^{o}C. The sample was pipetted into a 50ml plastic centrifuge tubes. The milk samples were defatted by centrifugation at 4,000 rpm for 10 min. Fatty layer was separated and filtered using whattman filter size 4, the resulting skimmed milk was transferred into a 50ml plastic syringe with a Luer tip which was attached to an immuno affinity column. The skimmed milk was allowed to flow into the column by gravity at a flow rate of approximately 2 ml/min. After the skimmed milk had run through, 20 ml of PBS was used to wash the column at a flow rate of 5ml/min. Air was passed through the column to remove residual liquid.

AFM_1 was eluted from the column at a flow rate of 1 drop/second with 1.25 ml of acetonitrile: methanol (60:40v/v) and 1.25 ml of HPLC water giving a total volume of 2.5 ml. One hundred (100) µl was injected into the HPLC system and AFM_1 in the final solution was determined using HPLC analysis. Equation for the amount of aflatoxin are made according to the following;

$$Wm = Wa \times \left(\frac{Vf}{Vi}\right) \times \left(\frac{1}{Vs}\right)$$

Where: Wm = Amount of aflatoxin M1 in the test sample in µg/ L
Wa = Absorbance corresponding to area of aflatoxin M1 peak of the test extract (ng)
Vf = Final volume of re-dissolved eluate (µL)
Vi = Volume of injected eluate (µL)
Vs = Volume of test portion (milk) passing through the column (mL) (Yohannes *et al.,* 2018)
Quantified levels of AFM1 from the sampling sites were analysed using ANOVA single factor and mean AFM1 were separated using Duncan's Multiple range

RESULTS

Table 1 shows result for screened raw cow milk samples for AFM1 using the rapid test kit specific for milk samples. Fifteen (15) samples were collected and tested. All the samples were contaminated with AFM1.

Table 1: Screened samples for AFM1 using rapid test kit from five (5) sampling sites

Location	No. of samples tested	No. positive
Dan-magaji	3	3
Gabari	3	3
Kufena	3	3
Hanwa	3	3
Tudun-wada	3	3
Total	15	15

Table 2: Quantified AFM1 levels from raw cow milk samples using HPLC analysis

Location	No. of samples tested	AFM1 in each sample (µg/L)	MeanAFM1 concentrations /location (µg/L)	No. above EU limit(>0.05µg/L)	
Dan-magaji	3	119.99 102.84 80.22	101.02[a]	3	p-value 0.000785 at 95%
Gabari	3	71.15 58.62 80.22	60.71[b]	3	
Kufena	3	79.28 62.34 47.26	62.96[b]	3	
Hanwa	3	38.09 26.22 30.52	31.61[c]	3	
Tudunwada	3	45.75 42.76 22.33	36.95[c]	3	

Statistically, there is significant differences between the level of AFM1 contamination and sampling locations. Calculated p-value is 0.000785 which is less than 0.05 at 95% confidence interval.

DISCUSSION

All the samples (100%) were contaminated with aflatoxin M1. This is the same with the reports of Maureen *et al*. (2019) in Kenya, the authors collected 96 raw milk samples and all the samples were contaminated with AFM1. The result is also similar to the reports of Elgerbi *et al.* (2004) and Fardos *et al*. (2017), the authors reported 71.4% and 74% level of AFM1 contamination in Jeddah and Libya respectively.

Quantified AFM1 levels obtained varied with locations, the variation may be due to differences in metabolic activities, type of feed given to the animals and the degree of AFB1 contamination in the feed given to the animals. All the samples were above EU limit. Location Dan-magaji had the highest level of contamination with an average of 101.02µg/L, this may be due to the fact that animals in that location are being fed with forage and cotton seeds, as cotton is among the products highly vulnerable to aflatoxin attack (Bhatnagar *et al.*, 2015). The mean in location Gabari and Kufena 60.71 and 62.96 µg/L respectively were similar as the animals in these two locations are being fed with maize fibre and forage. Maize fibre could be contaminated with AF as maize is highly susceptible to aflatoxin B1 attack (Anjorin *et al.*, 2016). Location Hanwa and Tudun-wada had the least with an average of 31.61 and 36.95µg/L, animals in these two locations feed on grasses.

Markaki and Melissari (1997) investigated the levels of AFM1 in commercial pasteurized milk in Greece and reported AFM1 ranging from 0.5 to 5ng/L. In Portugal, Martins and Martins (2000) studied the levels of AFM1 in 31 samples of raw and 70 samples of heat-processed milk, and found that 80.6% of raw milk and 84.2% of heat-processed milk samples were contaminated with AFM1. Among raw milk samples, 54.8% contained levels of AFM1 between 5 and 10mg/L and 19.3% had levels between 21 and 50mg/L. However, the occurrence of AFM1 in milk and milk products has been reported in the Mediterranean region including Egypt (Salem, 2002).

Pittet (1998) reported that concentrations of AFM1 in raw milk are usually less than 0.1ng/L in Europe, but might be greater than 1.0ng/L in other parts of the world. Varying levels of AFM1 in milk have been reported in surveys carried out in various parts of the world, although other factors might contribute to production of fungal toxins in food and feedstuffs.

CONCLUSION

Fifteen (15) fresh raw cow milk samples were collected and tested from five (5) different locations within Zaria metropolis. All the 15 samples screened were contaminated with AFM1. The samples were quantified using HPLC analysis to determine the extent of AFM1 contamination and all the samples were found to contain AFM1 at a level above EU set limit which was adopted by National Agency for Food and Drug Administration and Control (NAFDAC) in Nigeria. It is important to raise awareness and education on the health implications of aflatoxins to both humans and animals to the general public, identify appropriate technologies to control AFB1 attack in field and during storage of agricultural products and AFM1 contamination in milk and milk products.

RECOMMENDATIONS

1. It is recommended that dairy farmers adopt best pre- and post-harvest agricultural practices for crops used as animal feeds to curtail fungal colonization, toxin accumulation and subsequent contamination of animal milk in the local setting.
2. Farmers involved in milk production should be made aware of the adverse effects of aflatoxin contamination in animal feed. A systematic control program of supplementary feedstuff for lactating cows should be introduced by the public health authorities.
3. Regulatory agencies should employ adequate monitoring to ensure that AFM1 levels is below the set limit in milk and milk products

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