J. Trop. Resour. Sustain. Sci. 12 (2024): 123-129
https://doi.org/10.47253/jtrss.v13i2.1486

Journal of Tropical Resources and Sustainable Science

journal homepage: jtrss.org

Detection of aflatoxin M1 in raw cow milk in Perak district

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Received 9 January 2024
Accepted 30 June 2024
Online 23 December 2024

Keywords:
aflatoxin M1, raw cow milk, Perak district

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Abstract

Milk and dairy products are potentially contaminated with aflatoxin M1 (AFM1), especially in tropical and subtropical regions like Malaysia. This study aimed to detect the concentration of AFM1 in raw cow milk samples by ELISA method. A total of 46 samples of raw cow milk were received from four Milk Collection Centres (MCC) in Perak (A, B, C and D). 13.0% of the samples examined were found to exceed the European Commission (EC) 0.05 µg/kg maximum limit, with samples from MCC B and D accounting for 25.0% and 21.1% respectively. Statistical analysis showed that there was a significant difference in the mean concentration of AFM1 between the four MCCs at $p<0.05$. Among all these MCC groups, MCC A-B showed that there was a significant difference in concentration of AFM1 mean value at $p<0.0125$, while other groups of MCC groups were not significantly different. Nevertheless, all AFM1 concentration values in this study were below the 0.5 µg/kg limit set by the Food Act 1983 (Act 281) & Food Regulation (1985). However, the low concentration of aflatoxin observed in this study does not guarantee its absence in the future. Therefore, improvements especially in the process of handling and storing livestock feed and products (milk and meat) as well as consistent monitoring for the presence of aflatoxin to reduce the risk of aflatoxin toxicity to consumers.

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1. INTRODUCTION

Dairy products, especially milk, are an essential component of the human diet particularly for infants and young children (Rahimi et al., 2010). Due to high consumption of milk among the public any form of contamination of the milk is unacceptable to guarantee its safety and quality.

Aflatoxin M1 in milk occurs from the fermentation broth of *Aspergillus parasiticus* (Kamkar et al., 2014) and toxigenic strains of A. flavus (Iheanacho et al., 2014). Aflatoxins are toxic, carcinogenic, and/or teratogenic to humans (Sabbioni and Sepai, 1998) and animals (Raja and Harihara, 2005). Aflatoxin comes in six different forms; types B1, B2, G1, and G2 are found in plant-based meals, while types M1 (a metabolite of type B1) and M2 are present in meals derived from animals (Fink-Gremmels, 2008; USAID and Danya International, 2012). Aflatoxin contamination in milk and its products is produced in two ways; either toxins pass to the milk of cows, sheep, goats and buffaloes that have consumed feeds contaminated with aflatoxin B1, forming aflatoxin M1 through the metabolic process in ruminants and excreted in their milk or it results as subsequent contamination of milk and milk products with fungi (Arthur, 2012; Akeberegn and Almneh, 2019). By consuming tainted milk and other foods, humans can become infected with the toxin (Arthur, 2012). This hazardous substance created by fungi and molds is chemically stable and cannot be eliminated through the processing of food (Bezerra da Rocha et al., 2014). AFM1 is relatively stable in raw and processed milk products and cannot be destroyed by heat treatments pasteurisation, UHT technique and autoclaving (Motawee et al., 2004; Tavakoli et al., 2013). International Agency for Research on Cancer (IARC), 1993 categorised AFB1 as a class 1 human carcinogen and AFM1 as a class 2B possible human carcinogen (Cathey et al., 1994; Creppy, 2002; Galvano et al., 1996; Moss, 2002).

Climate influences contamination in part due to direct effects on the causative fungi. The complex communities of fungi that produce aflatoxin change along with the climate. This includes modifications to fungal community structure and changes in the quantity of aflatoxin-producers in the environment. Climate fluctuations can influence the predisposition of hosts to contamination by altering crop development by affecting insects that create wounds on which aflatoxin-producer proliferate (Cotty and Jaime, 2007). In hot, dry areas (latitudes between 30 and 40 degrees), aflatoxins are common (Lanyasunya et al., 2005) and particularly dangerous (Demissie, 2018) because these conditions are favourable for the growth of fungi that produce aflatoxin. The threat of aflatoxin contamination in food (groundnuts, tree nuts, maize, rice, figs and other dried foods, spices, crude vegetable oils and cocoa beans) is greatest in tropical and subtropical regions like Malaysia, where yearly

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average temperatures range from 28°C to 31°C (Abdullah et al., 1998) and there is a nearly constant presence of heavy rains. During the wet season, relative humidity ranges from 70% to 80%, and from 50% to 60% during the dry season (Sulaiman et al., 2007). Under these circumstances, commodities (peanuts, cereals, spices- chili peppers, black pepper, etc.) are extremely susceptible to fungal growth and develop mycotoxins and deteriorate quickly. The food chain could be contaminated at any point from cultivation through harvesting, storage, distribution, processing and consumption (Norhayati, 2000). To reduce or minimize the aflatoxin contamination in crops include rapid and proper drying, proper transportation and packaging, sorting, cleaning, drying, smoking, post-harvest insect control, and the use of botanicals or synthetic pesticides as storage protectants (Hell and Mutegi, 2011).

Aflatoxin associated with aflatoxicosis can cause acute and chronic poisoning. This toxin has teratogenic and carcinogenic effects to both humans (Sabbioni and Sepai,1998) and animals (Raja and Harihara, 2005). Acute aflatoxicosis resulted in death however, long term exposure or chronic exposure to low level/doses of aflatoxin M1 in dairy products may lead to liver cancer (Etzel 2002; Sherif et al., 2009), immune suppression, other reproductive disorders (Iqbal et al., 2013) and posing a significant human health hazard (Ismail et al., 2018; Peraica et al., 1999). Infants and children are the most vulnerable population due to their high milk consumption with raw milk and milk product (e.g., formulated cow's milk) and underdeveloped biochemical detoxifying mechanisms (Costamagna et al., 2019). Children that are exposed to aflatoxin for a long period may experience cognitive deficits, immune system suppression, and growth retardation. (Unnevehr and Grace,2013).

Many countries have implemented routine aflatoxin testing of dairy feed to reduce aflatoxin residues from entering the food chain (Volkel et al., 2011). Singapore for example, Singapore Food Agency (SFA) will randomly sample and tests food products sold in Singapore for mycotoxins. While in Malaysia, Ministry of Health (MoH), Malaysia only conducts continuous monitoring of foods that have notable risks to aflatoxin contamination and to ensure that the aflatoxin contamination is within the permitted level and the foods are safe for consumption (Victor Jeyaraj et al., 2022). Food Act (1983) and Food Regulation (1985), MoH, Malaysia have established the maximum allowable level of AFM1 is 0.5µg/kg. The maximum allowed level for AFM1 in infant formula was set by the Commission Regulation (EC) (2006) at 0.025 µg/kg and in raw milk at 0.05 µg/kg, respectively, while the US Food and Drug Administration (USFDA, 2000) set the standard for AFM1 in dairy products at 0.5 g/L.

Consumer awareness of food safety has led to research into advanced approaches and some innovative strategies to control mycotoxin contamination such as by using cold atmospheric plasma (CAP) (Kis et al., 2020; Hojnik et al., 2021), polyphenols and flavonoids (Mehany et al., 2021; Ahmed et al., 2022), natural essential oils (NEOs) (Cai et al., 2022) as well as magnetic and materials nanoparticles (Jebali et al., 2015; Luo et al., 2017). In Malaysia, studies by several researchers, such in Penang, Selangor and Terengganu, discovered aflatoxin contamination, in both fresh and processed milk samples.

In order to monitor the safety of our milk product, particularly in the Perak district, this study was conducted to measure the concentration of aflatoxin M1 in raw cow milk samples obtained from different Milk Collection Centres (MCC) in Perak. The ELISA (Enzyme Linked Immunosorbent Assay) method was used in this study because this technique having rapid output through user-friendly extraction method with high specificity (Velasco et al., 2003). ELISA is a widely used biochemical technique for the analysis of food safety and quality (Lu et al., 2017). It is also playing a crucial role in food production by providing rapid and accurate detection of allergens and mycotoxins, ensuring the safety and quality of food products. Their high sensitivity and specificity make them an indispensable tool for complying with regulatory standards and preventing food safety incidents (https://www.hygiena.com/learning-center/technology-guide/elisa). Although ELISA method was not a gold standard for determine AFM1 but study by Manggira et al., 2021 showed that the results obtained from ELISA kit were in agreement with those reported by the HPLC method, therefore it is suggested that ELISA can be considered as a reliable alternative to HPLC-FL.

2. MATERIALS AND METHODS

2.1 Milk samples

Forty-six raw cow milk samples were obtained from samples received from four MCCs in Perak (A, B, C, D). All the milk collected by MCC's were from regular dairy farmers which sent their milk samples for quality monitoring. The study was conducted in the Biochemistry Section, Veterinary Research Institute (VRI) using the ELISA method with each milk sample represented one dairy farmer.

2.2 Sample pretreatment procedure-Pretreatment of milk sample

A total of 1ml of milk samples were placed into 50ml centrifuge tube and was added with 4ml of acetonitrile. Mixture was then oscillated for 5 minutes and centrifuge at 4000rpm for 10 minutes at room temperature. 2.5ml of supernatant were placed to another centrifuge tube and was dried with water bath at 50˚C. The residual was vortex and mixed fully after being dissolved with 1ml reconstitution buffer. 50µl of milk samples were then be taken for analysis.

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2.3 Enzyme Linked Immunosorbent Assay (ELISA) Test

The concentration of AFM1 in this study was quantified by competitive ELISA technique using Aflatoxin M1 ELISA Kit (Elabscience, USA). Detection limit for this ELISA test kit was 0.1ppb.

2.3.1 Assay procedure

50µl of samples from pre-treatment procedure were placed into each well of ELISA plate and added up with 50µl of HRP conjugate and antibody working solution (50µl). The plate was covered with a sealer and gently oscillated for 5 seconds to mix thoroughly and incubated for 30 minutes at 25˚C in shading light. Next, the sealer was carefully uncovered and the liquid was removed. 300µl of wash buffer was immediately added to the each well. The wash procedure was repeated for 5 times, 30s interval each time. The plate was inverted and patted against thick clean absorbent paper. After the wash procedure, 50µl of substrate reagent A was added into each well and followed by 50µl of substrate reagent B. The plate was then gently oscillated for 5 seconds to mix thoroughly and incubated for 15 minutes at 25˚C in shading light. Later, 50µl of stop solution was added to each well and gently oscillated to mix thoroughly. The plate was then determined the optical density (OD value) of each well at 450nm (reference wavelength 630 nm) with microplate reader. This step completed within 10 minutes after stop reaction.

2.4 Data analysis

The concentration of AFM1 were calculated from absorbance reading by ELISA machine. Percentage of absorbance for each sample were identified based on formula below:

$$\text{Absorbance (\%)} = A/A_0 \times 100\%$$

The AFM1 concentration is determined by extrapolating the percentage absorbance of the sample against the concentration AFM1 of the standard (ppb). The results from the samples tested was statistically analysed using descriptive and non-parametric test in the Statistical Package for the Social Sciences (SPSS). The mean value of aflatoxin M1 was analysed based on significant differences at p<0.05.

3. RESULT AND DISCUSSION

A total of 46 raw milk samples from 46 dairy farmers were analysed with the competitive ELISA technique with eight (8) samples from MCC A, twelve (12) samples from MCC B, seven (7) and nineteen (19) samples from MCC C and D respectively. Thirteen percent (13%) of the samples tested were found to exceeded the maximum limit set by of the Commission Regulation (EC); 0.05µg/kg with 25.0% and 21.1% of the samples that exceeded the limit were from MCC B and D respectively. However, all the AFM1 concentration values in this study were below the maximum limit set by Food Act 1983 (Act 281) and Food Regulation (1985) at 0.5µg/kg. The mean concentration of AFM1 means value for MCC D was the highest (43.70±86.74) followed by MCC B (33.61±25.8), MCC C (17.56±8.3) and MCC A (6.01±6.4). The minimum rate of the concentration of AFM1 mean value was 0.0015 ppT while the maximum value was 389.7430 ppT. Based on the mean rank, MCC B (mean 28.71) had the highest average, followed by MCC D (mean 25.63), MCC C (mean 23.00), and MCC A (mean 11.06). The Kruskal-Wallis test was conducted to determine whether there is a difference in concentration of mean aflatoxin M1 value between the MCC. The results indicate significant differences, $X^2(3) = 9.165$, $p = 0.027$ therefore, the null hypothesis must be rejected and conclude that there is a difference in aflatoxin M1 values between the four MCCs (Table 1).

Table 1: Occurrence of AFM1 in four MCC in Perak

MCC	N	Mean±Sd	Mean Rank	Chi-Square*	df*	*p-value**
A	8	6.01± 6.41	11.06	9.165	3	**0.027**
B	12	33.61±25.83	28.71			
C	7	17.56±8.31	23.00			
D	19	43.70±86.75	25.63			
Total	46	30.53±58.29	-	-	-	-

*Significant different at p<0.05

Post hoc test Mann Whitney-Bonferroni corrected analysis were conducted to determine the mean concentration of AFM1 mean value among MCCs significant level of at p<0.0125. Among all these MCC groups, MCC A-B showed that there was a significant difference in the mean concentration of AFM1 means value, while there was no significant difference were observed in other groups of MCC groups (Table 2).

Table 2. Comparison of AFM1 mean concentration between MCC

MCC	A-B	A-C	A-D	B-C	B-D	C-D
p-value	**0.012***	0.020	0.012	0.150	0.5980	0.7071

*Significant different at p<0.0125

The first known case of aflatoxin exposure in Malaysia occurred in the 1960s when an epidemic of the disease at a pig farm in Malacca (Hamid, 1997) resulted in severe liver damage in the animals (Lim and Yeap, 1966). In 1988, there was a case of aflatoxicosis recorded in Perak, when 13 children died after eating noodles contaminated

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with 3 mg of aflatoxin (Chao et al., 1991). Wheat flour used to make the noodles was reported to contain aflatoxin due to the poor storage and processing, which in turn promoted the growth of pathogenic Aspergillus fungi and subsequently the production of aflatoxin (Lye et al., 1995). Since then, extensive research on aflatoxin has been carried out and the first monitoring was performed by the Institute of Medical Research (IMR), followed by other agencies such as The Food Technology Center (FTC), MARDI, and local universities (Hamid, 1997). A study conducted by Nor Shifa et al., (2016) on fresh cow milk samples marketed in Penang found that out of 102 samples tested, 4% were positive for aflatoxin M1 when tested using the High-Performance Liquid Chromatography (HPLC) method. In addition, a study by Siti Nabilah, (2014) on milk samples obtained from supermarkets in Puncak Alam Selangor found that from 69 samples tested, two (3%) were positive for aflatoxin M1 with concentration levels ranging from 0.036-0.045 ng/ml. Meanwhile, Farah Nadira et al., (2016) in their study of milk samples obtained from selected markets in Terengganu found that 19 out of the 53 samples (35.8 %) tested contained aflatoxin M1. However, in all of these studies, the aflatoxin M1 values obtained were still below the limit set by the Food Act 1983 (Act 281) & Food Regulation (1985) MoH, Malaysia.

Physical factors like pH, light, moisture, temperature, water, relative humidity as well as chemical factors such as atmosphere gases (nitrogen, oxygen, argon and other gases) are responsible for aflatoxin contamination (Abhishek et al., 2021). Therefore, the degree of contamination will differ depending on a region's agronomic and agricultural practices as well as the susceptibility of certain commodities to fungal invasion during pre-harvest storage and/or processing periods (Kim et al., 2000; Fung and Clark, 2004). Ding et al., (2015) reported that high moisture content always favours aflatoxin contamination because moist conditions are favourable for fungal growth and Thompson and Henke (2000), found that high humidity and temperature in tropical and subtropical regions than in temperate regions increase the susceptibility of crops to mycotoxin contamination compared to temperate regions. These studies demonstrate the crucial importance of environmental conditions such as moisture, humidity, and temperature in promoting fungal development and subsequent mycotoxin contamination in crops. Völker et al., (2013) stated that during daytime and in the summer, the cooling effect of ponds, lakes and rivers is between 0.5 to 4.8 K and the range of this effect is between some meters up to 400 meters away. The above statements support the finding of this study where 2 MCCs; MCC B, and D have high AFM1 concentration values. As it is known that, evaporation from open water bodies may lower the temperature, but on the other hand also increases the humidity (Albdour and Baranyai, 2019), and these conditions are favourable for fungal growth (Thompson and Henke (2000). The location of the 12 samples obtained from MCC B was found to be close to each other at 0.43 km and 0.2 km and approximately 0.4 km apart from water bodies like ponds and rivers. Whereas, the sample location from MCC D with the highest aflatoxin value (0.3ug/kg) is nearly encircled by water bodies with a radius of just 0.04 to 0.05km. However, Aflatoxin M1 levels in the current study were still below the Food Act 1983 (Act 281) and Food Regulation (1985) [Fifth Schedule (Regulation 39) - Microorganisms and Their Toxins (Table I & Table II)] as well as Codex Stan 193-1995 and the U.S. FDA which have a maximum limit of 0.50µg/kg.

According to a study conducted by Nile et al., 2016, cow's milk had the highest concentrations of AFM1 (62%) when compared with sheep, goat, and buffalo milk. Likewise, a study carried out in Iran by Ansari et al., (2019) showed that the amount of AFM1 contained in cow's milk was higher than the levels of both the EU and Iran. This indicates a possible health risk from consuming cow's milk that has been contaminated with AFM1 above regulatory authorities allowed limits. An assessment of aflatoxin M1 has also been carried out on fresh goat milk samples by Khairunnisak et al., (2018) who also found that the aflatoxin M1 value obtained was below the specified limit.

Besides dairy products, samples from other yields were also tested by other researchers, including raw peanut kernal and peanut-based samples (total aflatoxin) (Sahar et al., 2010; Mahror Norlia et al., 2018), cereals (total aflatoxin especially AFB1) (Hong et al., 2010), palm kernel cake (total aflatoxin) (Abdul Niefaizal et al., 2022), chicken feed (total aflatoxin) (Wan Syahidah et al., 2017) and urine samples (AFM1) (Siti Husna et al., 2021). Most of these samples were positive for aflatoxin and exceeded the standard limit. However, aflatoxin contamination can be controlled and prevented in order to reduce aflatoxin contamination by several methods, include use of resistant varieties, crop rotation, well-timed planting, weed control, pest control especially control of insect pests and avoiding drought and nutritional stress through fertilization and irrigation. Besides that, postharvest interventions should be done to reduce aflatoxin contamination by rapid and proper drying, proper transportation and packaging, sorting, cleaning, drying, smoking, post-harvest insect control, and the use of botanicals or synthetic pesticides as storage protectants (Hell and Mutegi, 2011).

4. CONCLUSION

In conclusion, all the AFM1 concentration values in this study were below the maximum limit set by the Food Act 1983 (Act 281) & Food Regulation (1985) at 0.5µg/kg. The low concentration of AFM1 value detected in the raw cow milk samples are reassuring, indicating the safety of the products for consumption. Although the

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concentration level of AFM1 in this study being within acceptable limits, the risk of AFM1 contamination remains high due to Malaysia's high relative humidity and warm climate, which are conducive for fungal growth. Thus, improving the handling and storage, as well as consistently monitoring for the presence of aflatoxin in dairy and livestock products, will ensure a reduction in the risk of aflatoxin toxicity to consumers. Improved management practices for territorial decisions, healthcare, and the dairy sectors may eventually result from the evaluation of the historical status and development trend in AFM1 scientific research. This evaluation can operate as a foundation for future study.

ACKNOWLEDGEMENTS

The authors wish to thank the Director General of Veterinary Services Malaysia, Director of Veterinary Research Division and Director of Veterinary Research Institute for permission to publish this article. We would also like to thank all the staff involves in this study for their help and support.

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