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Isolation of Fungal Species and Detection of Aflatoxin from Soy Milk Products using ELISA method

Maetrese Arianne J. Beley, Franco G. Teves, Ma. Reina Suzette B. Madamba
Dept. of Biological Sciences, College of Science and Mathematics, MSU-Iligan Institute of Technology, Lanao del Norte, PHILIPPINES

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Abstract

Fungal species isolated from the two batches of five randomly selected commercial soy milk samples were identified and found to be species of Aspergillus and Fusarium which are known to produce toxins detrimental when consumed at high levels particularly aflatoxin. Aflatoxin contamination in milk and other dairy products has been a serious worldwide problem has resulted in serious food safety and economic implications for the agriculture industry. Unfortunately here in the Philippines, despite the evolving dairy industry, only few studies have made about aflatoxin contamination. The aim of the study was to examine presence of aflatoxin on the milk samples were fungal species of Aspergillus and Fusarium was isolated, using Enzyme-Linked Immunosorbent Assay (ELISA) method. Milk sample 1 showed the highest amount of aflatoxin level with an average of 0.7 ppb. It exceeds the level of 0.5 ppb limit which was presented by the Food and Drug Administration in Table1. However, milk sample showed the lowest average amount of aflatoxin level with 0.6 ppb, but it still exceed the limit of 0.5 ppb. However, results must have to be first verified and analyzed to contribute to the additional information about the cases in aflatoxin contamination of milk products.

Keywords: *Aspergillus, Fusarium,* Aflatoxin, ELISA, soy milk.

Introduction

Aflatoxins are a group of structurally related toxic secondary metabolites and one of the most potent naturally occurring carcinogens or mutagens produced mainly by certain strains of *Aspergillus flavus* and *Aspergillus parasiticus*[1]. These filamentous fungi belong taxonomically to *Aspergillus* section *Flavi* including *Aspergillus oryzae* and *Aspergillus sojae*[2].

Food has always played an extra-ordinarily vital role in the rise and growth or the fall and decline of a nation because of its effect on the health of the population. Consumption of unsafe, contaminated food leads to food-borne diseases which cause considerable morbidity and mortality[3]. Aflatoxin contamination of foods and feeds is a serious worldwide problem resulting either from improper storage of commodities or pre-harvest contamination in corn, peanuts, cottonseed and tree nuts, especially during drought years. Harvested grains are colonized by various species of fungi, such as *Aspergillus flavus*and *Aspergillus parasiticus*, under such conditions leading to deterioration and mycotoxin production[4]. It was also reported that occurrence of AflatoxinM1 in cheese could probably increase the risk of developing cancer or toxic and carcinogenic effects. Moreover, fungi have been reported by Aissi et al. to cause extensive deterioration which may lead to the occurring of mycotoxinscarcinoms, mutagenicity and liver cancer[5]. Such contamination has resulted in serious food safety and economic implications for the agriculture industry. Because of the health concern, regulatory guidelines of 20 parts aflatoxin per billion parts of food or feed substrate (ppb) is the maximum allowable limit imposed by the U.S. Food and Drug Administration for consumption and for interstate shipment of foods and feeds.

Although aflatoxin biosynthesis has been documented for *Aspergillus flavus* and *A. parasiticus*, the closely related species *A. oryzae* and *A. sojae*, used in food and ingredient manufacture such as rice wine and soy sauce, no history of producing aflatoxins present in many strains of these moulds have recorded[2,6-8]. Therefore, there is concern regarding the potential expression of aflatoxin genes from *A. oryzae* and *A. sojae* undercertain conditions, since such expression could cause severe health problems. Many countries have carried out studies about the incidence of aflatoxicosis in milk. In most of them, samples have been found to exceed the limit imposed by many countries of 0.05 ug1-1. Unfortunately here in the Philippines, in spite of the fact that dairy industry has evolved a lot in the years as regards production levels and technology, there are few data bout aflatoxicosis incidence in milk, powder milk and other milky derivatives. The food industry requires strong evidence to prove the safety of the *A. oryzae* and *A. sojae* strains used in food production.

The quantity of aflatoxin level present on the milk samples was determined using Enzyme-Linked Immuno Sorbent Assay (ELISA) method by using the test kit which is a competitive enzyme immunoassay based on antigen-antibody reaction. ELISA is a powerful method for detecting and quantifying a specific protein in a complex mixture and enables analysis of protein samples immobilized in microplate wells using specific antibodies. The traditional methods of detection need around 1-

2 days for the target pathogen to be identified. Ever since these traditional methods evolved into molecular diagnostics rapid and accurate identification has been possible[9]. The technique has revolutionized immunology and is commonly used in medical research laboratories. ELISA also has commercial applications, including the detection of disease markers and allergens in the diagnostic and food industries.

Material and Methods

Fungal Samples: Fungal isolates were obtained from five branded and unbranded soya milk products available in retail outlets and brought to the Molecular Biology and Biotechnology Research Laboratory of MSU-IIT, Iligan City, Philippines, for analysis. Liquid milk samples were serially diluted and 0.1 ml was spread onto the surface of Potato Dextrose Agar (200 g potato infusion, 20 g dextrose, 15 g agar per 1000 mL of deionized water). 10g of the powdered milk samples were dissolved first in 100 ml of deionized water and was mixed for 5 minutes before it was diluted. Plates were incubated for 3-5 days for identification. *Aspergillus flavus* and *Aspergillus sojae* strains purchased from University of the Philippines-Diliman were used as positive controls for the identification of isolates. Streak plate cultures on Potato Dextrose Agar (PDA) medium were incubated at 27°C for five days. Isolated colonies were used for sub-cultures and for inoculation on PDA plates for genomic deoxyribonucleic acid (DNA) isolation.

ELISA Analysis: 5ml of each samples were placed to different sterile beakers containing 25ml of 70% MetOH, was put on a shaker for 2-3 minutes, filtered using a clean filter paper, before it undergone analysis using ELISA reader. Extracts were analyzed using Veratox® Total Aflatoxin ELISA Kit. A 100µl Conjugate was added to red-marked mixing well using 12-channel pipettor, then 100µl of controls and samples were added unto it. From the red-marked mixing well, 100µl was obtained and transferred to the antibody-coated wells, mixed three times and incubated for 2 minutes. Contents were then dumped into a waste container and the antibody-coated wells were washed 5 times with sterile distilled water. Excess water was tapped out on an absorbent paper towel. Next, 100µl Substrate from reagent boat was transferred to antibody-coated wells; mixed thoroughly and incubated for 3 minutes. Lastly, 100µl Red Stop solution was added to antibody-coated wells and mixed thoroughly. Results were interpreted using StatFax 303 ELISA reader.

Results and Discussion

Isolation of Fungal Samples: Five day old fungal colonies on Potato Dextrose Agar (PDA) of the randomly selected milk samples were carefully identified based mainly on the morphological characteristics such as the colony's color, form of conidia, presence of conidiophores, shapes of conidial heads.

Aspergillus flavus strain (figure-1A) was found to be isolated from samples 1 and 2 having woolly to cottony to somewhat granular texture, which are olive to lime green with a cream reverse in color and its microscopic morphology showed radiated conidial heads (figure-1E). *Fusariumsp.* strain (figure-1B) having a cottony-white appearance within the third day of the incubation period, which eventually became light brown and produces moist in days four to five of incubation was isolated from samples 4 and 5. However, *Aspergillus niger*which characteristically present dark-brown to black conidia, with uniseriate or biseriate conidiophores (figure-1C) was isolated from milk sample 5. Another strain fungi wasisolated from milk samples 1 to 4 and was identified as *Aspergillus parasiticus* (figure-1D) based on its massive dark green conidial head, with spherical spores as viewed under HPO (figure-1H). *A. parasiticus* is known to produce aflatoxins B_1, B_2, G_1 and G_2[10] and is economically known to be important in the production of citric acid in which it is one of the most efficient, highest yield bioprocesses currently used in the industries[11].

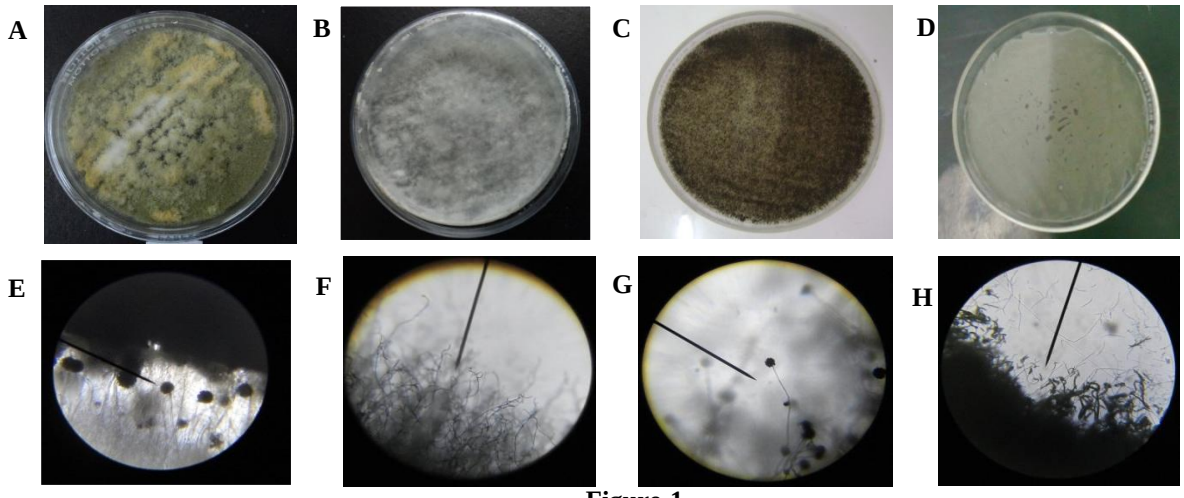

Figure-1
Fungal species isolated from the five soy milk products with its corresponding LPOmicroscopic image viewed under light microscope.(A) *Aspergillus flavus* (B) *Fusarium sp.* (C) *Aspergillus niger* (D) *Aspergillus parasiticus*

ELISA Analysis: Aflatoxins are the only mycotoxins currently regulated by the Food and Drug Administration and established action levels which prohibit the use of contaminated products[12]. These toxins are of particular public health importance because of the diseases they cause to humans. Ingestion of higher doses of aflatoxin can cause acute aflatoxicosis, or in severe cases, liver failure[13]. Hence, contamination of these toxins on food stuffs is of great concern in rural communities because of its effects. Table 1 shows the acceptable levels of aflatoxin in food and feed recommended by the US Food and Drug Administration.This guideline served as the basis in determining if the randomly selected milk samples collected, qualifies the aflatoxin level presented.

Table-1
Food and Drug Administration guidelines for acceptable levels of total aflatoxin in food and feed

Action level	Commodity	Species
0.5 (aflatoxin M_1)	Milk	Humans
20.0	Any food except milk	Humans
20.0	Feed	All species

Milk samples were analyzed so as to determine the level of total aflatoxin present. This was done using Veratox ELISA Kit and results were interpreted using StatFax 303 ELISA reader.Veratox for Aflatoxin is a competitive direct ELISA that provides a quantitative analysis of aflatoxin in such commodities as corn, cornmeal, corn gluten meal, corn/soy blend, wheat, rice, milled rice, milo, soy, whole cottonseed, cottonseed meal, raw peanuts, peanut butter and mixed feeds.

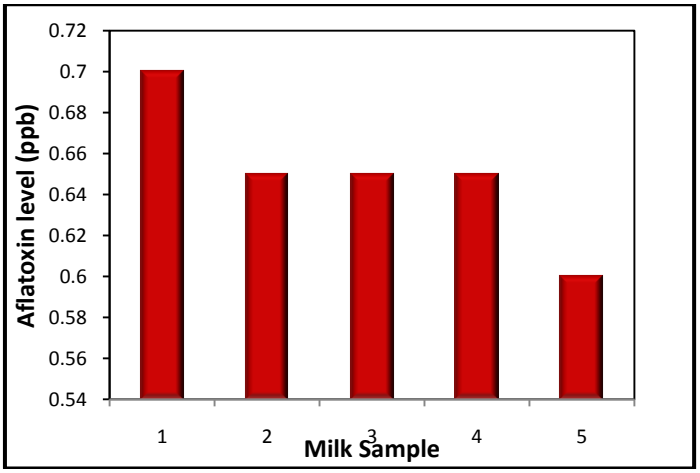

Figure-2
Average aflatoxin level in two batches of six soy sauce samples detected using Veratox ® ELISA Kit and StatFax Elisa Reader

The preparation procedure in the immunoenzymatic methods is very simple as it consists mainly in extraction and there is no need for purification or isolation of the tested component. The above results showed that considerable amount of aflatoxin levels are present in the soy milk samples. Milk sample 1showed the highest amount of aflatoxin level with an average of 0.7 ppb. It exceeds the level of 0.5 ppb limit which was presented by the US Food and Drug Administration in Table1.However, milk sample showed the lowest average amount of aflatoxin level with 0.6 ppb, but it still exceed the limit of 0.5 ppb. Milk samples 2, 3 and 4 on the other hand, showed an amount of aflatoxin level ranging from 0.64-0.65 ppb approximately.

Conclusion

Competitive direct enzyme-linked immunosorbent assay (CD-ELISA) test is one of the most common tests conducted in immunobiochemistry that allows the user to obtain exact concentrations in parts per billion. On the study, test resultsshowed considerable amount of aflatoxin levels are present in the randomly selected soy milk samples. It is able to advance plausible predictions and accelerate research on detection of aflatoxin level of the basic commodity used for human consumption, such as milk. It is of great help to ensure that aflatoxin levels of the commodities used for human and animal consumption are controlled and thus should not exceed the levels higher than 4 to 15 ppb.However, although the advanced prediction of aflatoxin level appears clear, experimental verification will have to be done to corroborate the ELISA results. These are molecular identification of the isolated fungal species paired with database searches and improved software tools that could give a better understanding of the biosynthesis and biological activities of aflatoxin producing fungi, isolation of the aflatoxin regulatorygene from local strains of *Aspergillus sp*., sequence comparison with homologues from other *Aspergillus sp.* isolates in databases, and phylogenetic analysis.

Acknowledgements

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