Trends in Life Sciences
An International Peer-reviewed Journal
ISSN: 2319–4731 (p); 2319–5037 (e)

ASPERGILLUS CHEMOTAXONOMY (II) ;FUNGAL AFLATOXIN G (G_1 AND G_2) PRODUCTION PATTERNS IN 24 DIFFERENT ANAMORPHIC SPECIES OF NORTHERN IRAN ISOLATES LABORATORY CULTURE MEDIA

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ABSTRACT

Mycotoxins are metabolites of fungi capable of having immunotoxic-carcinogenic effects in man and animals. Contamination with mycotoxins is a major problem of food and feeds storage which leads to adversely effects also economic losses influencing the public health and agriculture. While contamination by mycotoxigenic fungi is a major problem, and serum-based methods work on‘‘one substance one assay’’ concept as alternative approach, ELISA was used to identify directly these molds before the toxin production and then cheked by HPLC.Identification and specification of Aspergillusisolates from food and feed or environmental samples have resulted in development of more and more chemical technologies or molecular approaches.. We have started to diverge the attention toward the molecular technology in the clinical, forensic science and regional agriculture sector in response of toxigen isolates nich by conducting more than 100 isolates obtained from northern provinces of Iran containing 24 distinctive species using ELISA/HPLC immunoassays in a double blinde investigation on whole 10 day old culture substrate resijuesrespectively. According to the findings of the examination of the correlation between aflatoxin values were measured by ELISA to measure the relationship between the Countercurrent out of the all cases (sig:0.00,z:- 621/3) and numerically significant difference exists between them,comparisonly between the average aflatoxin G_1 and aflatoxin G_2 obtained by HPLC method there was not significant differences in the mean values of aflatoxin G_1 and G_2despite of countercurrent relationship which consider these as ‘gold standard’ with reliable specificity and sensitivity regarding to FAO/WHO level definition by µ-ng/kg-g.Identification and typing of Aspergillus from cultures or environmental samples have resulted in development of more and more chemical technologies or other molecular approaches.

KEY WORDS: *Aflatoxin-G, Aspergillu ,Iran, Molecular detection*

Introduction

Mycotoxins are secondary metabolites produced by different wellknown species of fungi,cause of different economical damage in veterinary/public health and industrial agriculture. At first, mycotoxin exists in mycelium of poisoning fungus and later the growing substrates/medium as well

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as spore of organisms is known as poisoning factors(Zheng et al, 2005). The potentiality of Aflatoxin's poison depended on different factors such as the toxin types,dosage, duration , nutritional situation, metabolism, and immune mechanisms that is affected by Aflatoxin causes carcinogenic, cytotoxicity, mutation damage. Occurring/presence of the Aflatoxin in dietary and increasing concentration from critical limits(5-20 ppb in diet and 0.5 ppb in milk) cause the decrease of general ability in human and animal Determining (Gary, 2005). Aflatoxin has different detecting methods like Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC), Gas Chromatography (GC), and immunochemical methods such as Enzyme Linked Immunosorbent Assay (ELISA). HPLC is ideal and more useful than the other methods in terms of specificity (0 – 320 ppb) and sensitivity of this process.Aflatoxins are currently the most studied due to larger impacts onperformance and therefore greater economicimpact as secondary toxic metabolites produced by certain fungioccurence as natural contaminants and causativedisease and mortality (Kabar, 2006). Determination of biochemical toxic effects of aflatoxins isimportant for diagnosis of toxicosis.

Materials and Methods

From the May - October 2010-2011, sampling was done using settle plates based on CBS rules according to "CBS" instructions from indoor and outdoor stations.One sample group was taken from agricultural area fields and also per each processing plant too. Six plates including Malt extract agar(MEA),Yeast extract agar(YEA),Czapek,s agar(CZA),Czapeks Yeast extract agar(CZYA), Saboraudsdextrouse agar(SDA) and Potato dexterous agar (PDA), all with 100 ppm chloramphenicol were applied then removed after 30, 60 and 90 minutes for outdoor and 15, 30 and 60 minutes for indoor sites, respectively.All plates were incubated at 25°C aerobically, then examined in the periods of 3,7,10 and 14 days to identify any growings so that they were harvested,subcultured,marked and then cultivated in the conserving prepared plates.Finally,for macroscopic and microscopic morphology examinations,107 aspergillus colonies were cultivated and grown at 25°C in order to identify and rank the colonies, various conventional mycological methods were used based on the ICPA rules for morphologic and microscopic and macroscopic examinations.In all of the samples, micrometry and photography were done by Leica system for micro analysis microscope.All isolates were cultured in 50ml Falcon tubes containing Czapek,s broth medium at 25°C aerobically in a shaker incubator adopted for 200rpm for 10days. Afterward fungal biomasses harwested then dried out in dessicator Then, the samples were converted to powder by pearl/vortex and the initial culture media separated so that to be passed from plesezec number 20 [5,7,22,23]. the samples were packaged in plastic pocket to be away from any moisture that may cause growing fungi and increasing the amount of Aflatoxin [3,17,23].

ELISA determination

To detect aflatoxin levels in the fungal biomasses and the culture medium samples using the Competitive ELISA Procedure as described by R-Bio-Pharm GmbH was used and measured at the absorbance of 450nm (Rosi et al, 2007).

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HPLC determination

In the analytical procedures of aflatoxin analysis by HPLC, were done in three steps: extraction, purification or cleaning up and quantitative determination [16]. In order to do HPLC process,the centrifuged samples were analyzed by using the immunochemical kits after that to be filtered through filter paper and at least collecting the volume of 25 ml from each samples. Furthermore, the samples were waighed (25g) plus 2.5g salt and 100ml exciting solution were collected and mixed/passed to the filtration paper then were passed through an immunoaffinity column (C18 column Supelco Discovery® 250× 4.6mm I.D., 5mm particle diameter) at a flow rate of 1 drop/sec, which were applying vacuum if it would be needed (vacuum system). The column was washed twice and was dried by moderate pressure of air. According to the test instructions ,extraction of toxin was done by using methanol solution and water. Initially, we injected extracts to the columns. The column includes gels containing antibodies specific for Aflatoxin B and G variants. The aflatoxins were released by adding 500 ml of extracting solution (methylacetic nitric)remaining inside the column for 20 min, and then 1500 ml of distilled water was added. At the next step, the column was washed by PBS solution. This leads to remaining B and G Aflotoxin which attached to gel and removal of other materials of cell extract. Then, the column was washed by special ethanol solution. The elution (2000ml) was filtered through a Millipore filter (0.45mm), which was injected into the HPLC system[19]. The mobile phase including one of water, methanol, acetonitrile, phosphate buffers or a mixture of them, was entered in the column and poured into the device bottle. Then the detector was turned on and set to 360 nm wave length and the electronic recorder switched on and speed of mobile phase current justified based on RP. Finally, the device was placed in the current, for 30 to 45 minutes without injection. After 45 minutes, absorption of the mobile phase was stopped, system suitability test was added and the results recorded. As a result, resolution factor, middle peaks, tailing factor, number of sub-pages of the column for measuring test material (theoretical plates), and capacity factor were calculated. To realize the detection level of aflatoxins which are based on fluorescent(B_2/G_2)/non fluorescent(B_1/G_1) character in HPLC system, therefore, banded together by potassium bromide compound in mobile phase solution was completely used post column derivatization chamber with electrical flowing and the ionization1computer graph is drawn in accordance with the passing time (prevention time of aflatoxin B_1/ B_2 and G_1/G_2 were 11,9,8 and 6). the standard solution was injected 5 times to the device and its scale value was obtained. This value was bigger than %98. Then each test standard was injected 3 times and reference standard solution injected, afterwards. Finally the device compared average of the results of the samples curve areas with average of the results of standard curve areas and showed the effective material value based on ppb. Samples graph, standard graph and the kind of aflatoxin were determined and measured (ppb) based on calibration and Samples graph, standard graph and the kind of aflatoxin were determined and measured (ppb) based on calibration graph [19]. In this trial, we utilized k2 independence test and Anova table, which are used to show correlation or irrelation of two classified variables.

Results and Discussion

Geographical distribution of 107 conducted samples followed: 68 samples (%63) are from Gilan, 30 ones (%23) from Mazandaran and 9 ones (%8) from Golestan, with the least share of Genus Aspergillus isolates. According to the results, Elisa method can evaluate total amount aflatoxins on the other hand HPLC system determines the types of aflatoxin and measuring them. Generally, there are two tests for measurement of aflatoxin: Rapid test and quantity test (quantification test). In many laboratories, after recognizing that the samples were infected, they used rapid method. Usually the cost of rapid test is 1.3 to 1.7 of the complicated tests. Rapid test including immunoassay tests, such as ELISA, was used in general and semi specificity laboratories. However, the best suggestion is using inevitable test after rapid test to determine high level of affection by specific mycotoxin that must be implemented in this situation[1,2,18].

In comparison, the average of total aflatoxin estimated by a competitive ELISA method in fungal culture media arranged by 0-2ppb produced by *A.flavous,A.fumigatus,A.niger*and *A.nidulans* isolates while the higestaflatoxin producer were *A.ostianus*(8-10ppb,mean4ppb) incontrast the lowest levels(less than 2ppb) belonged to many different isolates. The average value of aflatoxin G_1 calculated in culture medium samples was showed a peack of 0-1ppb that has been produced by fungi such as *A. niger*, *A. flavus*, *A. fumigatus* and *A.candidus*isolates.The minimum value of Aflatoxin G_1 was more than 0-0.2 ppb and the average of its maximum is less than 5ppb(1-3ppb) and the average of aflatoxin G_2 in culture medium was 0-0.1ppb too, even the highest examined amount were in the range of 0-0.2ppb which is produced by *A.niger*.In culture medium the average of aflatoxin of G_1 and G 2 which is measured by HPLC, showed a correlation and there was a meaningful numerical difference (Sig: 0.000, pc: 0.930). According to the fact that numerical differences resulted from toxin measurement in statistical examinations has been meaningful, so we can find out that except the confirmed correlations and non alignment between them, the numerical differences regarding to error tolerance of commercial kits could be reasonable and this led to the meaningful differences.In examining meaningfulness of the numerical differences and the kind of correlation between average values of whole Aflatoxin G1 and G 2 ,each one separately measured by HPLC, no statistical correlations between the cases was seen and also no significant correlation despite reverse or non alignment relation, it could pointed to above cases. According to the findings of the examination of the correlation between aflatoxinvalues were measured by ELISA to measure the relationship between the Countercurrent out of the all cases (sig:0.00,z:- 621/3) and numerically significant difference exists between them.Comparisonly between the average aflatoxin G_1 and aflatoxin G_2 obtained by HPLC method there was not significant differences in the mean values ofaflatoxin $G_1$and G_2while consistent Countercurrent relationship.This study showed that not only sensitivity/specificity of HPLC system is more than ElISA method but also HPLC system is the only way to determine the realy concentration of aflatoxins variants with more sensitivity, determining the small amounts of lowconcentratsaflatoxin. There was no significant difference between two methods during this study at a level of ppm values. However, On the other hand, the measureable rate of aflatoxin with HPLC was 0-320 ppb The comparison of.these two methods showed that HPLC system had specific ability to measure the concentration of aflatoxin. B_2, G_1 and G_2 but Elisa kit detected just total concentration of aflatoxin$B_1$and can be kept one year maximally in 18 – 30˚C but HPLC system has a different advantages in this situation. In HPLC system, the method in which immunoaffinity column is used the column poured excited juice to measurement of antibodies against aflatoxin. This system has B_1, B_2, G_1, and G_2. The aflatoxin poison attached to the antibodies that were located in column and other particles. Finally,

suitable solutions (methanol) were pass through the column and the aflatoxin poisons were separated and also the level of aflatoxins were detected. Important characteristic of HPLC system is its sensitivity and specificity in recognizing the amount of aflatoxin in comparison with the Elisa method. Meanwhile, Elisa kit is faster,cheaper and easier to do to use than HPLC system (Papp et al, 2002).

Acknowledgment
With special thanks to The Research and Technology deputy of the Islamic Azad University, LahijanBranch for finantialand good support and assistance for this work.

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