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ORIGINAL ARTICLE

Aspergillus chemotaxonomy (I); fungal biomass aflatoxin G production patterns in 24 different Anamorphic Species of Northern Iran isolates

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ABSTRACT
Cosmopolitan genus Aspergillus can be harmful in indirectly mycotoxin production, a major problem of food and feed products or storages which can lead to adversely effects (aflatoxicosis) also economic losses influencing public health and agriculture. The naturally predominant occurring toxins designated as aflatoxin B_1, B_2, G_1, and G_2 are of the most toxic carcinogenic secondary metabolites and aflatoxicosis causative agents concomitantly. Therefore, molecular methods in combination with certain techniques can possibly be used for the molecular detection of Aspergillus and its metabolites in an efficient way. Since aflatoxins are believed to produce by three phylogenetically distinct sections it is crucial to determine distribution of toxigen populations by high performance inspective detection relatively rapid and highly sensitive are inclosed of quantitative detection. 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 blind investigation on whole cell extract respectively. Likewise the others ,we discriminate the aflatoxin-producing(47 samples, more than30IU) from the non-aflatoxigenic through the same process(43 samples, under 5-10IU)and even some strain as"fast"(41 samples,more than 30IU) or "slow" aflatoxin accumulators(16 samples, under 30IU) 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 the development of more and more chemical technologies or other molecular approaches. We will suggest approaches to investigate more efficient on the matter of aflatoxin contamination of crops and food products in the conducted areas.
Keywords: Aflatoxin-G,Aspergillus,Iran,Molecular detection.

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INTRODUCTION

Aflatoxins are secondary toxic fungal metabolites produced as *Aspergillusflavus* and *A. parasiticus*. There are four naturally occurring aflatoxins, the most hepatotoxic being aflatoxin B_1 (AFB_1), and three structurally similar compounds, namely aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and Aflatoxin G_2 (AFG_2). Aflatoxins not only contaminate our food stuffs but are also found in edible tissues, milk and eggs after consumption of contaminated feed by farm animals [1]. Aflatoxin, a potent hepatotoxic and hepatocarcinogenic mycotoxin, induce lipid peroxidation in rat liver and associated with various diseases such as aflatoxicosis and hepatocellular carcinoma [2]. An epidemiological survey indicates that the occurrence of hepatic and kidney disorder is increasing as lifestyle changes causing serious problem in the area of public health. Swine are highly susceptible to aflatoxins. Extreme effects can lead to death, but the greatest impact comes from reduced reproductive capability, suppressed immune function, reduced productivity capability and various pathological effects on organs and tissues [3]. The objective of this study was to determine the effects of aflatoxin induced toxicity in the liver and kidney of albino rats.

MATRIALS 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), Saborauds dextrose agar (SDA) and Potato dextrose 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, subcultures, 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 morphological and microscopic and macroscopic examinations. In all of the samples, micrometry and photography were done by the Leica system for micro analysis microscope. All isolates were cultured in 50ml Falcon tubes containing Czapek, s broth medium at a 25°C aerobically in a shaker incubator adopted for 200 RPM for 10days. Afterward fungal biomasses harvested, then dried out in a desiccators Then, the samples were converted to powder by pearl/vortex and the initial culture media separated so that to be passed from places number 20 (9,29). The samples were packaged in plastic pocket to be away from any moisture that may cause growing fungi and increasing the amount of aflatoxin [14, 29].

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 observance of 450nm (21).

HPLC Determination

In the analytical procedures of aflatoxin analysis by HPLC, were done in three steps: extraction, purification or cleaning up and quantitative determination (12 &19). In order to do the 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 of each sample. Furthermore, the samples were weighed (25g) plus 2.5g salt and 100ml exciting solution was 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 we're 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 with PBS solution. This leads to remaining B and G aflatoxin which attached to gel and removal of other materials of cell extract. Then, the column was washed with a special ethanol solution. The elution (2000ml) was filtered through a Millipore filter (0.45mm), which was injected into the HPLC system (21 &22). 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, the 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 fluorescent (B_2/G_2) /nonfluorescent (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 aflatoxinB_1/B_2andG_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 sample curve areas with the average of the results of standard curve areas and showed the effective material value based on Pbb. Sample graph, standard graph and the kind of aflatoxin were determined and measured (Pbb) based on calibration and Sample graph, standard graph and the kind of aflatoxin were determined and measured (Pbb) based on calibration graph [5, 21]. In this trial, we utilized K2 independence test and ANOVA table, which are used to show correlation or relation of two classified variables.

RESULTS AND DISCUSSION

Geographical distribution of 107 conducted samples followed: 68 samples (%63) are from Gilan, 30 once (%23) from Mazandaran and 9 once (%8) from Golestan, with the least share of Genus *Aspergillu*s isolate. According to the results, ELISA method can evaluate the total amount aflatoxins on the other hand an 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 tested after rapid test for using inevitably determine high level of affection by specific mycotoxin that must be implemented in this situation [22,23,24]. In comparison, the average of total aflatoxin estimated by competitive ELISA method in fungal biomasses arranged by 20-25ppb produced by *A. flavors, A. fumigatus, A. sage* and *A. nidulans* isolates, while the highest aflatoxin producer were *A.ostianus* (40-45ppb) in contrast the lowest levels (less than 5ppb) belonged to many different isolates such as *A. flavors, A. fumigatus, A. niger*and *A. nidulans*. The average of aflatoxin G1 determined using an immunoaffinity HPLC method in biomass showed by the maximum peak of 1ppb which were produced by fungi such as *A. niger, A. flavus,A.fumigatus*and *A.candidus* where as the average of aflatoxin G_1was0.05ppb in contrast according to the average of aflatoxin G_2 with HPLC method in biomass the highest examined amount was in the range of 0-1ppb which is produced by fungi such as *A.niger, A.flavus*also the average ,maximum and minimum of aflatoxin G_2 with HPLC method was 0-1ppb.In examining meaningfulness of the numeral difference and the kind of correlation between average values of whole aflatoxin G_1 and G_2 each one separately measured by HPLC, no meaningful numerical difference between the cases was seen and also no significant correlation despite reverse relation, we can point to above cases.

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 really concentration of aflatoxins variants with more sensitivity, determining the small amounts of low concentrates aflatoxin. 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 0-320ppb [25,26,27]. 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 B_1concentration of aflatoxin 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 aflatoxin. This system has antibodies against 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 cheaper and easier to use than HPLC system [7,29,30].

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