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A Study on the Potential of Ochratoxin Production by Aspergillus Sp. of Gilan, Mazandaran and Golestan Provinces and Ochrotoxin Rate of Producing Grown Cell Biomass in the Laborating Conditions

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ABSTRACT

Objective: Molds produce poisons called mycotoxins,OTA is one of the most relevant mycotoxins which is generally produced by Aspergillus, its presence in food and feed products being regulated in many countries. **Methods:** From the first May to the last October2011,sampling was done according to"CBS" instructions for indoor and outdoor situations. Isolates were identified and recorded in the laboratory on fungal biomass prepared a sample group and crude cell extracts were prepared in the laboratory and Ochratoxin was produced by fungal isolates in cell extracts was measured by using ELISA. **Results:** Significance rate is higher than 0.05.Thus the changes in Ochratoxin production don't depend on various levels of geographic area ,it can be stated that in the Aspergillus species studied in this geographic area,it is expected to observe Ochratoxin production in 2.5-5ppb range. **Discussion:** According to the fact that maximum limit toxin in food stocks is 5 ppb,it must be greatly noticed into consideration.While it must be taken greatly into attention that some of isolated studied in the area produced the toxin in great amounts (20-25ppb).That may be dangerous for health significance of correlation between the toxin rate and the original province where sampling of studied isolates was performed is 100% for Guilan and Mazandaran and 99% for Golestan provinces.It may be stated that it is not possible to find as specific area with considered field conditions to be the growth area or origin of a given Aspergillus species with a given density of their presence or their dependence to a area or dependence of their classification the section of subgenus groups in the standard rules for classification of Aspergilli.

1.INTRODUCTION

Molds are filamentous (fuzzy or dusty appearing) fungi that occur in many feedstuffs, including roughages and concentrates.which can infect dairy cattle, especially during stressful periods when they are immune suppressed, causing a disease referred to as mycosis. A wide range of different poisons called mycotoxins that affect animals when they consume contaminated feeds. Molds produce poisons called mycotoxins that affect animals when they consume contaminated Molds are present throughout the environment and therefore, mycotoxins can be formed on crops in the field, during harvest, or during storage, processing, or

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feeding.eeds.The mycotoxins of greatest concern include: Aflatoxin, which is generally produced by Aspergillus mold; deoxynivalenol, zearalenone, T-2 toxin, and fumonisin, which are produced by Fusarium molds; and Ochratoxin and PR toxin produced by Penicillium molds.The discovery of aflatoxins in the 1960s (Sargeant et al.,1961), with approximately 100,000 turkey poult deaths in England, was the seminal event that made the scientific community realize that mold secondary metabolites could be responsible for food and feed safety problems. Several other mycotoxins were identified when fungi in food were more fully investigated. Ochratoxin A (OTA) was purified and characterized from Aspergillus ochraceus Wilh. Strain K-804 (van der Merwe et al.,1965) isolated from sorghum grain, and proved to be acutely toxic to Pekin ducklings, mice and rats (Frisvad et al.,2006). Nowadays, OTA is one of the most relevant mycotoxins, with its presence in food and feed products being regulated in many countries. OTA is composed of a 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3-R-methylisocoumarin (Ochratoxin α) moiety and a L-β-phenylalanine molecule, which are linked through the 7-carboxy group by an amide bond. The compound belongs to a group of fungal secondary metabolites,commonly known as Ochratoxins, which share a similar chemical structure. Ochratoxin B(OTB), Ochratoxin C (OTC), ochratoxin α (OTα), ochratoxin β (OTβ), (4-R)- and (4-S)-hydroxy-Ochratoxin A (4R-OH OTA and 4S-OH OTA), 4-hydroxy-Ochratoxin B (4-OH OTB) and 10-hydroxy-Ochratoxin A (10-OH OTA) are naturally produced by certain fungi.OTA is produced by several Aspergillus species , like (Aspergillus section Circumdati, *A. cretensis, A.flocculosus, A.melleus, A.ochraceus, A.ostianus, A.persii, A. petrakii, A. pseudoelegans,A. roseoglobulosus, A.sclerotiorum, A. steynii, A.sulphureus, A.westerdijkiae Aspergillus section Flavi, A.alliaceus ,Petromyces albertensis(Petromyces alliaceus), Aspergillus section Nigri, A.carbonarius, A. lacticoffeatus, A.niger,A.sclerotioniger, P.nordicum* and *P.verrucosum* and *P.nordicum*.These penicillia are the species most authoritatively listed as OTA-producing. The *P.viridicatum* strains that were reported as OTA producers were reclassified as P. verrucosum by Pitt (Serra et al.,2006). Other species such as P. chrysogenum, P. brevicompactum*, P. crustosum, P. olsonii* and *P. oxalicum* have been claimed as OTA producers (Frisvad et al.,2006, Hesseltine et al.,1972). Nevertheless, a careful confirmation of these findings is required since no other authors report the capacity of these species to produce OTA. Strains of a larger number of species are known to produce OTA in Aspergillus. Species above accepted by Frisvad and co-authors.Some other species were reported incorrectly as OTA producers, due to either misidentification of the isolates in question or to the use of contaminated cultures. Others still were reclassified, having now a different scientific name. For example, the original A.ochraceus strain from which OTA was first isolated, was reclassified as A.westerdijkiae, and many other A.ochraceus strains as *A. steynii* (Bayman et al.,2002).The major OTA producers in food and feed products are considered to be *A. alliaceus*, *A. carbonarius*, *A. ochraceus*, *A. steynii, A. westerdijkiae*, *P. nordicum* and *P. verrucosum* (Varga et al.,1996). These are mainly associated with agricultural crops pre-harvest, or in post harvest storage situations. P. nordicum is a high OTA producer and is isolated predominantly from certain cheeses and fermented meats. Aspergillus niger, which is a very common species, is less relevant since most of the isolates are non-Ochratoxigenic (Serra et al.,2006, Pitt et al.,2001) and the others usually produce small amounts of OTA (Esteban et al.,2004). However, since it is a widely used species in several biotechnological processes it can pose some safety issues in the manufacture of food grade organic acids and enzymes. The other OTA producers are rare and do not pose a great concern for food safety.The average European consumption of grape-juice is not known, but Swiss data indicate that it is very low, about 5 ml/day. However, its Ochratoxin A content might be of concern since it is consumed by young children. Consequently, a high consumption rate has been assumed, but not in The overall daily intake of Ochratoxin A calculated in Table 1 is 3.5 ng/kg bw. This figure coincides well with the SCOOP assessment, which was in the range 0.7 to 4.6 ng/kg bw. According to the General Standard for Contaminants and Toxins in Foods, MLs shall only be set for those foods in which the contaminant may be found in amounts that are significant for the total exposure of the consumer. In CX/FAC 98/13 it is suggested that a significant amount should contribute to more than 10% of the total dietary exposure(Samson et al,.2004). Enzyme-Linked ImmunoSorbent Assay (ELISA) is an antibody-based assay that is commonly used to detect mycotoxins(Michael et al.,1961). A number of commercial ELISA kits are available for Aflatoxins, deoxynivalenol, fumonisins, ochratoxin, and zearalenone. This is usually a competitive assay in which the mycotoxin of interest from a sample competes with a labeled mycotoxin for a limited number of specific antibody-binding sites. The greater the amount of toxin present in the sample, the lower the binding of the labeled toxin and the lower the signal generated by the assay (CAST.,2003). ELISA is one of the more affordable methods for detecting mycotoxins, and has the advantage that a large number of samples can be measured at once. Commercial 96-well assays and strip-tests are available for many mycotoxins.ELISA techniques have been shown to be less accurate and sensitive than conventional chromatographic assays. Very few correlations were found between the two types of techniques. In addition, false positive or false negative results often occurred with ELISA because of cross-reactions between molecules or interferences with the antibody reagents. They are thus considered to be suitable for qualitative assessment or for sample pre-screening but not for quantitative determination. It is also recommended to

use the ELISA techniques only for the foods they were developed for.Customers of Canada's grain use technology that can test for Ochratoxin A in the parts per billion range. To put that into perspective, one second in 32 years is one part per billion.Fortunately, data collected each year from our monitoring program show that the levels of ochratoxin A in Canada's grain are generally below international limits. We want to help you keep meeting these low limits.

Table 1.

Ochratoxin A limits for various countries (www.grainscanada.gc.ca.title of Prevent ochratoxin A in stored grain.,2011)

Ochratoxin A limits for various countries - Current as of April 2011		
Country	**Product**	**Ochratoxin A limit (ppb)**
Iran [1]	Wheat	5
Israel [1]	Cereals, cereal products	50
Switzerland [1]	All foodstuffs	5
Turkey [1]	Raw grain	5
	Food made from grain	3
European Union [2]	Unprocessed cereals	5
	All products made from unprocessed cereals, intended for direct human consumption	3
China [3]	Cereals	5
	Legumes	5
USA [3]	FDA has not set advisory limits or action levels for ochratoxin A in any commodity	

2. MATERIALS AND METHODS

From the first May to the last October 2011, sampling was done according to"CBS" instructions for indoor and outdoor situations. One sample group was taken from among 50000 meter square area fields and also per processing, plant using settle plates based on CBS rules too. Six plates, including Malt extract agar (MEA), Yeast extract agar (YEA), Czapek s agar (CZA), Chapek s Yeast extract agar (CZYA), Saborud s dextrose agar (SDA) and Potato dextrose agar (PDA), all with 100 ppm chloramphenicol and 50 ppm tetracycline were applied for one sample group. All the plates were incubated at 25±2 c aerobically then examined in the periods of 3,7 and 15 days . At last ,107 colonies were cultivated for macroscopic and microscopic morphology examinations the study on the morphology and macroscopic features, front and back colors, pigments , umbrella , foundings and grown masses and also examining and micrometery was done by microphotometic estroscope and microscope, the samples with the help of slide culture prepared then were done by leica micro analysis microscope hard and soft wares. All samples prepared as mentioned above for an indirect Competition ELISA assay for fine quantitation of total Aflatoxin based on the manufacturer's instructions.For all samples and standards. of estimated and then corrected datas reflecting to standard curve obtained as ELISA reader calibrated by 450 nm UV the light for comparing the density of samples and standard OP and preparing final results.

3. RESULTS

In this study on the mean Ochratoxin produced among the studied species, the greatest produced toxin was related to the species *A.melleus* subgenus *Circumdati* section *Circumdati* and amount of produced toxin was 20.1473 ppb.The second species in respect of mean produced toxin was related to *A.parasiticus* of subgenus circumdat branch Flavi with 4.1951 ppb toxin amount. The third species is related *SP3(A.af.nomius*)subgenus circumdati section Flavi with3.2675 ppb production rate. The fourth species is related to *A.sojae* subgenus circumdati section Flavi with3.2579 ppb toxin production rate. The fifth species is related to *A. terreus* subgenus *Nidulantes* , section *Terrei* with 3.2579 ppb toxin production rate. The sixth species is related to *A. af.nidulans* subgenus *Nidulantes* , section *Nidulantes* with 3.2551ppb toxin production rate. The seventh species was *A.ochraceus* subgenus circumdati branch circumdati with 3.2515 ppb toxin production rate.The eights species was *A.ostianus* subgenus circumdati section circumdati with 3.2515 ppb toxin production rate. The ninth species was *A. sp vI* subgenus *Non* section *Non* with 3.2491ppb toxin production rate.The tenth species was *A.awamori* subgenus circumdati section *Nigri* with 3.2459 ppb toxin production rate.The eleventh species was *S. ornata* subgenus *Oranati* section *Oranati* with 3.2459ppb toxin production rate.The twelfth species was *A.carbonarius* subgenus *Circumdati* section *Nigri* with 3.2315ppb toxin production rate.The thirteenth species was *A.flavus* subgenus *Circumdati* section *Flavi* with 3.2115 ppb toxin production rate.The fourteenth species is *A. fumigatus* subgenus *Fumigati* section *Fumigati* with 3.0333ppb toxin production rate. The fifteenth species is *A.candidus* subgenus *Circumdati* section *candidi*, with 2.8355ppb toxin production rate.The sixteenth species is *A. niveus* subgenus *Nidulantes* section *Flavipeds* with 2.4997ppb toxin production rate.The seventeenth species is *A.alliaceus* subgenus *Circumdati* section *Flavipeds* with 1.5273ppb toxin production rate.

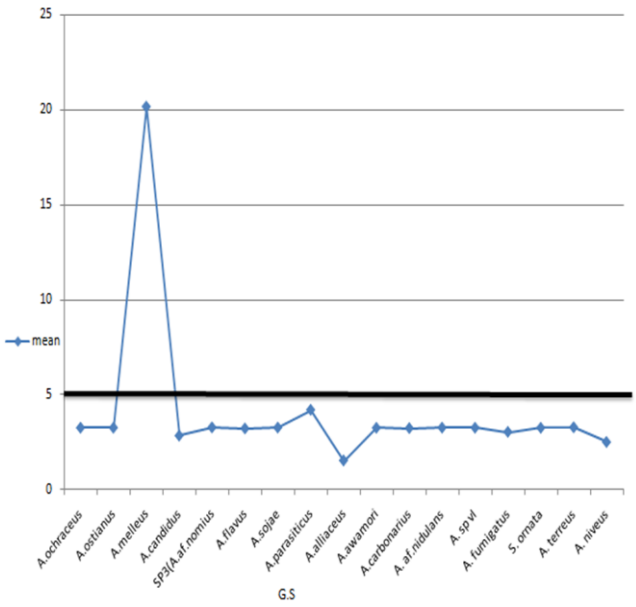

Figure 1. Mean measured toxin and how the toxin is produced by Aspergillus sp. Identified in the studied samples.

According to the figure (2) among 17 Aspergillus species isolated from Northern parts of Iran 14 species (*A.ochraceus*،*A.ostianus*، *A.candidus) SP3(A.af.nomius*، *A.flavus* ،*A.sojae* ،*A.parasiticus* ،*A.awamori*،*A.carbonarius* ، *A. af.nidulans*، *A. sp vI*، *A. fumigatus* ،*S. ornata*، *A. terreus*)have produced the toxin with 2.5-5 ppb range, with 82.3 % ratio. Few number (2 species) including *A.alliaceus* , *A. niveus* have produced the toxin ranging 0-2.5 ppb(11.7%). Followingly one species (*A.melleus)* has produced the toxin at 15.5-20 ppb range(5.8%). According to the result, among 17 isolated species , 14 species including (*A.ochraceus* ،*A.ostianus* ،*A.candidus* ، (SP3)*A.af.nomius* ،*A.flavus* ،*A.sojae* ،*A.parasiticus* ، *A.awamori* ،*A.carbonarius* ،*A. af.nidulans* ،*A.spvI* ،*A. fumigatus* ،*S.ornate* ،*A. terreus* among Aspergillus species isolated from northern part of Iran produced the greatest amount of toxin ranging 5-7.5 ppb with 82.36 % which is twice the toxin standard and then they produced the toxin ranging 22.5-25 which is five- fold the toxin standard. A.melleus with the ratio 5.8 % in the range of 7.5 -10 ppb A.alliaceus and in the rane of 0-2.5 ppb , A. niveus have produced the toxin.(figure3). According to the figure, among 17 species 16 species including *A.ochraceus* , *A.ostianus, A.candidus, SP3(A.af.nomius), A.flavus, A.sojae,A.parasiticus,A.alliaceus,A.awamori A.carbonarius* ,*A. af.nidulans*, *A. spvI* ,A. Fumigates ,S. ornate ,*A. terreus* wre from Aspergillus species(ranging -5.5-+5.5 ppb)which toxin productions were in extremely low rates Near zero rate . A species *A.melleus* had the lowest toxin production rate ranging +5.5-20 (with 5.89 % ratio) (figure4).

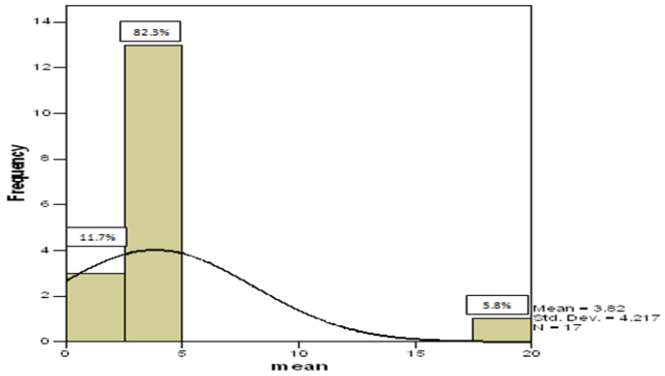

Figure2. frequency and distribution of isolates producing mean toxin based on toxin production potential.

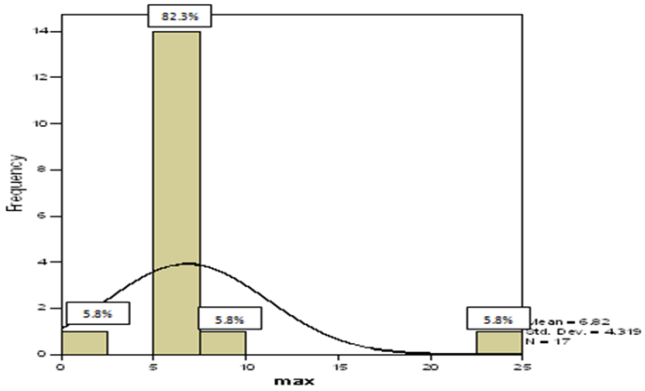

Figure 3. frequency and distribution of isolated, producing the greatest amount of toxin

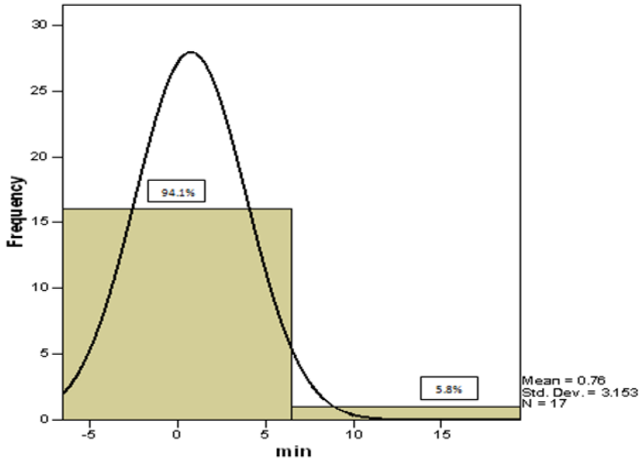

Figure 4. frequency and distribution of isolated producing the lowest amount of toxin

According to the datas , the greatest number of isolates was 6 isolated related to the indoor region from sheltered sampling areas with 2.84 minimum , 20.15 maximum and 6.001 mean. In the samples obtained from plain and interstitial areas , the number of isolated was 4 with minimum 2.50, max=3.27 and mean 3.06. In the plain fields, there was two isolates with 3.25 mean. In the interstitial and forest areas , there were 2 isoltes with 3.03 minimum, 3.25 maximum , 3.13 mean. In the plain , interstitial and forest areas there was 1 sample with 3.21 minimum, 3.21 maximum , 3.21 mean.(Table1).At test result indicates , significance rate is higher than 0.05. Thus the changes in Ochratoxin production don't depend on various levels of geographic area. (Table2).

Table 2.

mean toxin produced in each geographic area

Sheltered area 0=

1=plain area

2=interstitial

3= forestry

outdoor	Mean	N	Std. Deviation	Minimum	Maximum	Range
0	6.0010	6	6.93229	2.84	20.15	17.31
1	3.2519	2	.00849	3.25	3.26	.01
3	2.8612	2	1.88642	1.53	4.20	2.67
12	3.0636	4	.37625	2.50	3.27	.77
23	3.1396	2	.15033	3.03	3.25	.21
123	3.2115	1	.	3.21	3.21	.00
Total	4.1163	17	4.16388	1.53	20.15	18.62

Table 3.

ANOVA for mean toxin produced in each geographic area

			Sum of Squares	df	Mean Square	F	Sig.
mean * outdoor	Between Groups	(Combined)	33.117	5	6.623	.298	.904
	Within Groups		244.289	11	22.208		
	Total		277.406	16			

4. DISCUSSION AND CONCLUSIONS

In the statistical analysis performed in present study , frequency subgenera recognized are indicated where greatest frequency was related to subgenus Circomdati . including *A.alliaceus* , *A. niveus* have produced the toxin ranging 0-2.5 ppb(11.7%) . Followingly one species (*A.melleus)* has produced the toxin at 15.5-20 ppb range(5.8%).Based on our analysis, statistical frequency related to the identified sections are indicated where the greatest frequency was related to the section Flavi .Aspergillus meleus with 22.5-25 ppb had the greatest amount of produced toxin which is more than 4 times higher than the acceptable limit. In contrast, Aspergillus aliceus with the lowest mean rate significantly differed from other studied species with 2.5-5 ppb mean ochratoxin pattern. Flowingly, Aspergillus niveus had the lowest amount of produced toxin in 0-2.5 ppb range. Aspergillus melleus and Aspergillus parasiticus had the highest amount of toxin production . However, maximum toxin production by Aspergillus parasiticus ranges between 7.5-10 ppb and it was still more than the maximum range of toxin production of other species I. e. 5-7.5 ppb. This amount for Aspergillus alliaseus and A.niveus ranged between 0-2.5 ppb.According to the figure 3-9 , it can be found that the mean value and maximum normal amount of toxin increased, on average, up to 5 ppb and the curve is skewed to the right. While figure 3-10 , skewed to zero, due to very high number and having zero minimum in the measurement of minimum normal toxin curve among other species.Thus, it can be stated that in the Aspergillus species studied in this geographic area, it is expected to observe ochratoxin production in 2.5-5 ppb range, according to the fact that the maximum acceptable limit for this toxin in the food stocks is 5 ppb, it must be greatly noticed into consideration. While it must be taken greatly into attention that some of isolated studied in the area produced the toxin in great amounts (20-25ppb). That may be dangerous for health (figure 3-10,3-11,3-12).According to table 3-10 , numerical differences of toxin produced by the number of isolates taken in Gilan and followingly in Mazandaran and finally in Golestan provinces had the greatest correlation and significance. Thus, it can be stated that among the isolates obtained in the geographic area of Gilan province (z: -3.621), it is possible to isolate more diverse species with more toxin production ability with greater numbers. In this property, Mazandaran province due to datas (z: -3.527) is placed with some distance after Golestan province(z::-3.479) which is placed in situe with much more distance and probability tend to be decreased. Significance of correlation between the toxin rate and the original province where sampling of studied isolates was performed is 100 % for Guilan and Mazandaran and 99 % for Golestan provinces. In the study of the total size and distribution of samples obtained in 3 studied provinces it was recognized that with $p < 0.06$ and z: -1:833 there is significant relationship between mean measured ochratoxin and the number of distributed samples. Sig : 0.68, z:-1.823. Thus, it is not possible to define or recommend a given area or province as a more safe region or more risky area (table 3-10). It may be stated that it is not possible to find as specific area with considered field conditions to be the growth area or origin of a given Aspergillus species with a given density of their presence or their dependence to an area or dependence of their classification the section of subgenus groups in the standard rules for classification of Aspergilli (figure 3-13). Simultaneous Results of our study, on the presence of studied samples in both the field area(outdoor) and in the closed area(indoor) of food processing centers indicate that there is not a significant difference between the presence of achieved species or specificity of a given species to a given field or covered area($p>0.05$).

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