A Study on Properties of Deoxynivalenol (DON) Production in Culture Medium by Aspergillus Spp. Isolates from Northern Iran

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ABSTRACT

The increased fungi contaminations and related damages cause disease through production of toxins in animals and thus in humans. Since they are not easily distinguishable, then it is crucial to study their characteristics. Aspergillus are among the most important toxigenic fungi that are found abundantly in northern Iran habitat which is one of most important habitat of Iran and is the main source for many feed and food stuffs in the state. Hence, we aimed to study on properties of deoxynivalenol (DON) production in culture medium by 24 Aspergillus spp. isolates from northern Iran. Samples were collected from Northern Alborz and Southern Caspian Sea agricultural plants cultivation areas and processing centers. Samples were then isolated and identified based on CBS environmental sampling rules and ICPA diagnostic standards. They were cultured to stimulate the toxin production until the targeted toxin to be measured at culturing substrate and fungi biomass. Afterward, they were exposed to extraction and existing DON size were measured by ELISA technique Our results indicated that in addition to Fusarium, genus Aspergillus has a potent ability to produce DON toxin or alike molecules. However, validation of this issue needs further studies, in particular, by using advanced biochemical or genomic molecular techniques.

Keywords: Culture medium, Deoxynivalenol (DON), Aspergillus spp

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INTRODUCTION

The genus *Aspergillus* belongs to the class Euascomycetes of the Phylum Ascomycota. The genus *Aspergillus* includes seven subgenera, each containing several species. *Aspergillus* colonies grow rapidly, producing white, green, yellow, or black colonies (Verweij PE and Brandt ME, 2007) as a group of molds that are ubiquitous in nature and common natural environments are resistant to many disinfection treatment methods. In fact, *Aspergillus* can quickly flourish at sites of biofilm development (Geldreich EE, 1996 and Warnock DW, 2012). *Aspergillus* spp. are found worldwide, and widely distributed in the environment (Ryan KJ, 2004 and Araujo R et al., 2006). *Aspergillus* and *Penicillium* are economically, ecologically, and medically too much important. As a large genera, *Aspergillus* species are commonly found as contaminants in foods during drying and subsequent storage (Pitt JI et al., 2000 and Samson RA et al., 2002).

Mycotoxins are low molecular weight secondary metabolites produced by certain strains of filamentous fungi such as *Aspergillus*, *Penicillium* and *Fusarium*, which mainly invade crops in the field and may grow on foods during storage under favorable conditions of temperature and humidity (Reddy KRN et al., 2010 and Iheshiulor OOM et al., 2011).

Mycotoxins produce carcinogenic, immunotoxic, neurotoxic and teratogenic effects in susceptible consumers (Dwivedi P et al., 2008). Over 100 different fungal species produce mycotoxins (Trefilov PV, 2011). Of which the greatest concern include: aflatoxin B1 which is generally produced by *Aspergillus* mould, deoxynivalenol (DON), Zearalenone, T-2 Toxin, and Fumonisin B1 which are produced by *Fusarium* moulds, ochratoxin A and citrinin which are produced by *Aspergillus* and *Penicillium* moulds are regularly implicated in toxic syndromes in animals and humans (Dwivedi P et al., 2008). No region of the world escapes the problem of mycotoxins or mycotoxic syndrome with estimation that there are about 300 harmful mycotoxins (Iheshiulor OOM et al., 2011). Problems associated with mycotoxin contamination are generally complex. These compounds are highly chemically stable and very difficult to denature. Aflatoxins, ochratoxins, cyclopiazonic acid as well as fusariotoxins such as zearalenone, deoxynivalenol, moniliformin and fumonisins, are common, and all of them can be found in pet foods (Böhm J, 2006). Mycotoxins causes mycotoxicoses and their toxicity depends on the amounts ingested, exposure time-span, species- breed susceptibility,

age, sex, health status, but also other parameters such as density, food-feeding preferences, diseases and environmental conditions like relative temperature or humidity (Gams W et al., 1998). Consumption of food or feed contaminated with mycotoxins may cause various diseases defined as mycotoxicoses which are immediate menace to human and hematothermal animals (cows, pigs, dogs, cats etc.) (Korbas M and horoszkiewicz-Janka J, 2007). Different types of mycotoxin-producing fungi notably develop in different weather conditions, and therefore types and levels of mycotoxins in cereal grains may vary significantly (Trefilov PV, 2011). DON is one of the least toxic reliable mycotoxins, however, it is the most prevalent trichothecenes in human foods and its presence is an indicator of the possible incidence of other more toxic trichothecenes (AlHazmi NA, 2011). They cause significant economic losses in animals due to reduced productivity, increased disease incidence, chronic damage of vital organs and decreased reproductive performance. The Figure 1 shows the chemical structure of DON (Murthy KK et al., 2009 and Iheshiulor OOM et al., 2011).
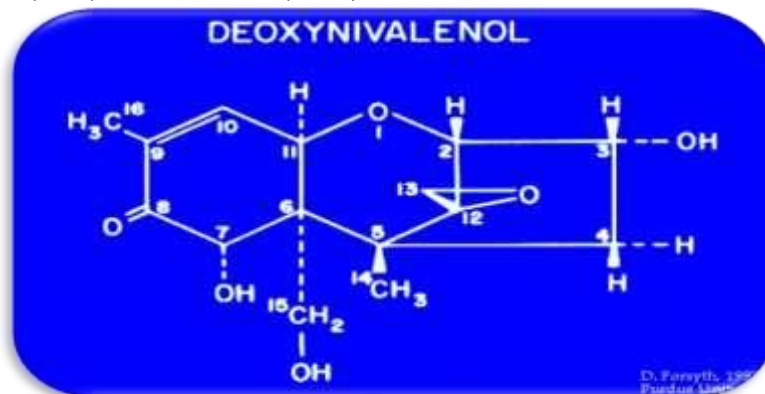

Figure 1: Chemichal structure of Deoxynivalenol (DON/Vomitoxin)
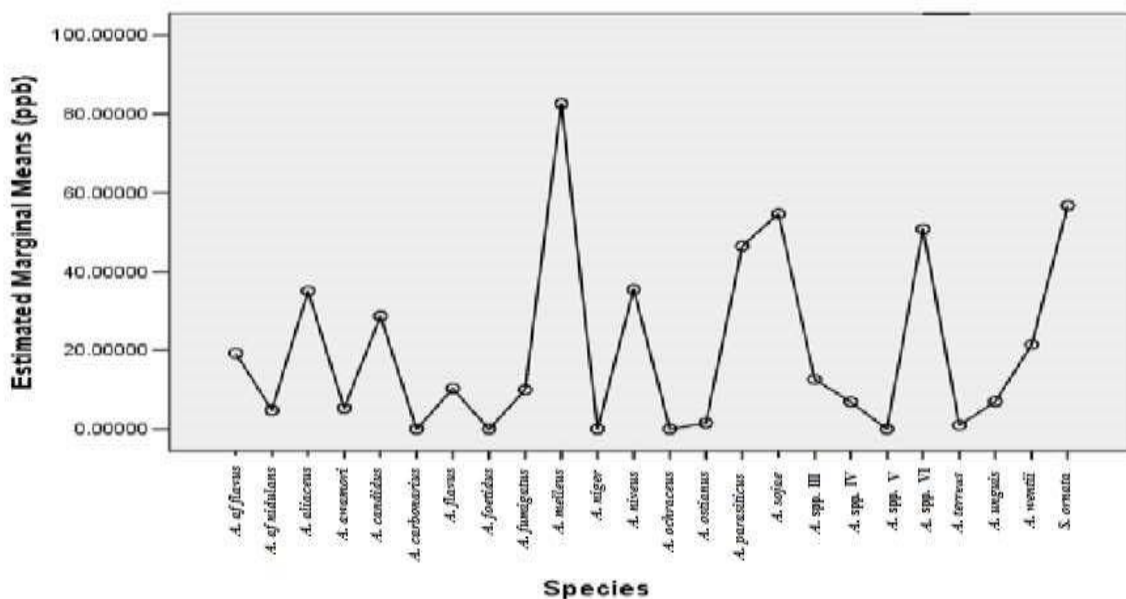

Figure 2: Showing deoxynivalenol (DON) production mean in culture medium based on species
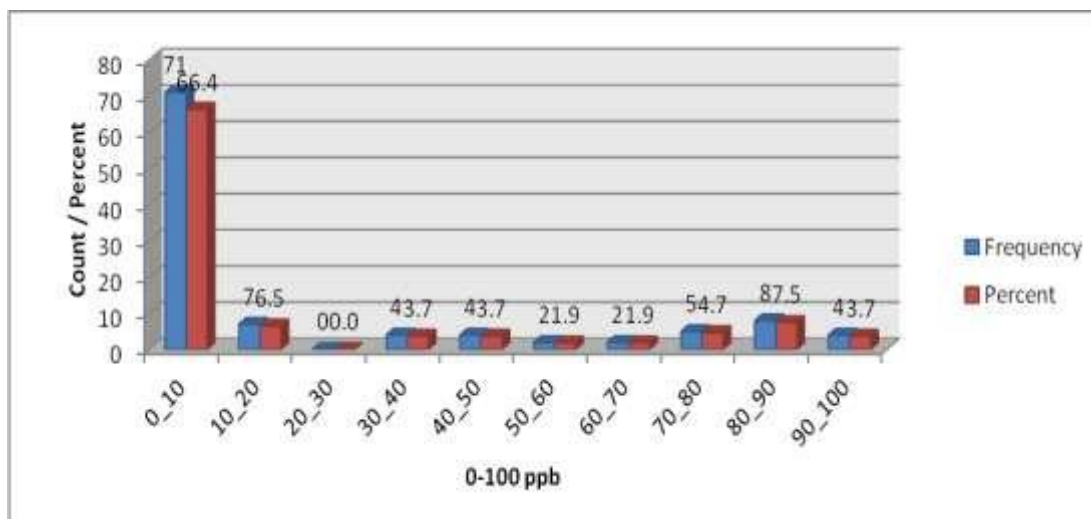
Figure 3: Showing the frequency of isolates based on deoxynivalenol (DON) concentration in 0-100 ppb intervals
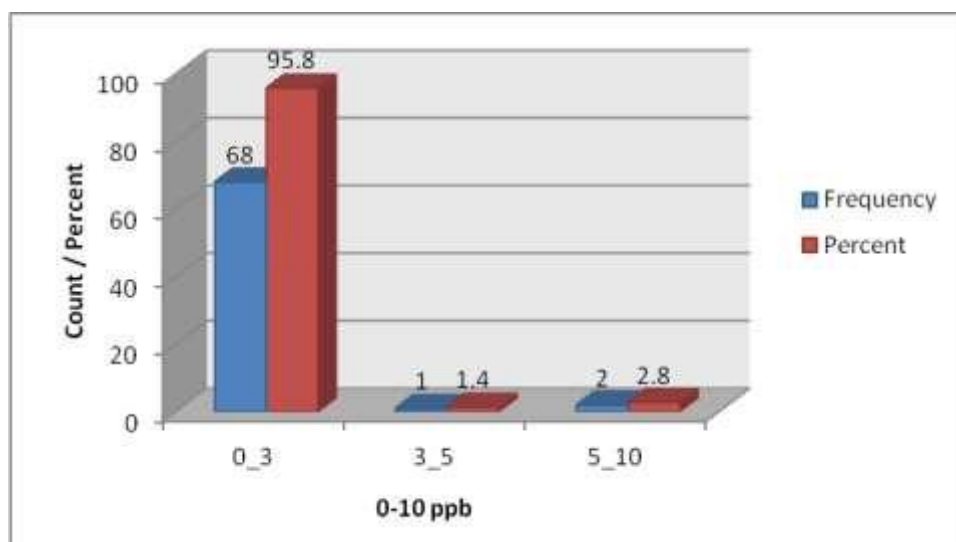
Figure 4: Showing the frequency of isolates based on deoxynivalenol (DON) concentration in 0-10 ppb intervals

As these mycotoxins are ubiquitous, the testing of products is required to keep our food and feed safe. For this purpose, sensitive and reliable tests are needed to detect contaminations. One detection possibility is an immunoanalytical based test which needs antibodies as reagents (Korbas M and horoszkiewicz-Janka J, 2007 and Baumgartner S et al., 2010). Hence, we aimed to study on properties of DON production in culture medium by 24 *Aspergillus* spp. isolates from northern Iran

MATERIALS & METHODS

Sampling, Culturing and Isolation

Sampling was done from indoor and outdoor (based on CBS institute sampling program) from May until mid of October in Gilan and Mazandaran (Kozakiewicz Z, 1989; Samson RA et al., 2001; Klich MA, 2002 a). Sampling was done by a group of six plates having Malt extract agar (MEA), Yeast extract agar (YEA), Czapeck yeast extract agar (CZYEA), Czapeck agar (CZA), Saburaud dextrose agar (SDA) and potato dextrose agar (PDA) containing 100 ppm chloramphenicol and 50 ppm tetracycline for extracting one sample group (Kozakiewicz Z, 1989; Pitt JI and Hocking AD, 1997; Samson RA et al., 2001; Klich MA, 2002 a; Klich MA, 2002 b). Plates were harvested

(with 15-25 cm³ of agar and 10-12 cm diameter) after 30, 60 and 90 minutes (451 suitable plates in the outdoors) and 15, 30 and 60 minutes (441 suitable plates in the indoors). We removed them after putting their lids, marking and enclosed them with meshy polyethylene bags before sending to laboratory. All plates were incubated aerobically in 25±2 °C (Odds FC et al., 1983; Samson RA et al., 2001; Klich MA, 2002 a). For 15 days in the intervals of 3, 7 and 15 days, all plates usually (and also daily) were examined to identify new grown colonies which were visually seen or were seen with stereomicroscope. Then they will be marked and picked up with a strile glass niddle and cultured in pre-prepared multipurpose agar filled plates (Kozakiewicz Z, 1989; Gams W et al., 1998; Samson RA et al., 2001; Klich MA, 2002 a). All new-found moldy samples subcultured and incubated based on previous program and every macroscopic and microscopic characteristics were followed in the intervals of 5, 10 and 15 days and recorded (Kozakiewicz Z, 1989; Samson RA et al., 2001; Klich MA, 2002 a). Finally, from 300 *Aspergillus* colonies (from more than 600 generative moldy isolates), 150 colonies were selected and cultured in CZDA, CZYEA (with and without 20% sucrose), MEA and CZDA (with and without 20% sucrose) for morphological micro and macroscopic studies due to ICPA taxonomic rules (Kozakiewicz Z, 1989; Samson RA et al., 2001; Klich MA, 2002 a; Klich MA, 2002 b).

Morphological Studies

For morphological studies and micro and macroscopic imaging, the surface and back of one to two weeks mature colonies (in black *Aspergillus* of two to four weeks colonies) were selected. Measuring the colonies diameter, examination of the color of surface and back of colonies, pigments, any extrolits together with aspergils, cells and grown masses, filaments, stipes, corona of conidia and micrometry of conidiophores, vesicles of conidia and examination of the any generations and micrometric imaging of sclerotia or asci were done with stereoscopes linked to Leica® Microanalysis Software Shared Network System (Kozakiewicz Z, 1989; Samson RA et al., 2001; Klich MA, 2002 a). In all samples with slide culture, tease mount and steaky tape of conidiophores (stipe), vesicle, corona of conidia, phialides, metullas, conidia or asci and all accessories of them, micrometry or imaging were done consequently (Kozakiewicz Z, 1989; Powell KA et al., 1994; Gams W et al., 1998; Samson RA et al., 2001; Klich MA, 2002 a).

Providing media samples/ Cellular extracts

To provide prepared isolates extracts, CZ liquid medium + 2% ME was selected for cultivation and more toxination. A loop full (10⁵ phialospores) of PBS-conidies suspension of each grown isolates in Czapeck extract agar (CZEA) plates were picked up and inoculated into one 50 ml falcon tubes containing Czapeck broth (CZB) with 2% malt extract (Shadzi S, 1993). Then will be incubated with 200 rpm, in 25±3 °C

with a natural photoperiodic light-darkness conversion while were examined daily to inhibit any moldy matt on the liquid even in the third and sixth days by adding liquid medium with 1% MEA until usually 50 ml liquid medium to be remained in the test tubes (Odds FC et al., 1983; Green BJ et al., 2003; Oda K et al., 2006). After seven days, floating or deposit fungi floating masses that were small and new borne (Germ tubes) moldy fungal short filaments centrifuged in 3000 rpm 15 minutes till to be precipitated and harvested (Ausubel FM et al., 2002).

Recognition and estimation of DON were done by direct ELISA method using RIDASCREEN ® DON (Art. No.: R5906) which is a competitive enzyme immunoassay for the quantitative analysis of DON in feed and foods. All valuable and trustable data were analyzed by varieties of statistical softwares using as Excel (version 2007) and SPSS (version15). (IBM Corp, 2012)

RESULTS

Chi-Square test was used to study the effect of geographic criteria and distribution on frequency of species. Our result showed that there was not significant correlation between frequency of species and the geographic criteria and distribution as shown in Table

Table 2 and Figure 2 show the deoxynivalenol (DON) production mean in culture medium based on species. ANOVA test was used to study the effect of species on DON production mean. Our data showed that there was significant correlation between species and DON production as shown in table 3

Table 4, 5, 6 and 7 show the brief introductive toxin producer isolated species based on DON acceptable maximum value and Feed & Food reliable international standards. Our results showed that the West of Mazandaran with a mean 29.810 ppb had the highest DON production potency followed by the East of Gilan with a mean of 20.274 ppb. The West of Gilan (15.971 ppb) had the lowest toxin mean rate.

Table 8 and Figure 3 show the frequency of isolates based on DON concentration in 0-100 ppb intervals. Our results showed that the 0-10 ppb interval with a frequency 71 (66.4%) had the highest prevalence. The 50-60 and 60-70 ppb interval with a frequency 2 (1.9%) had the lowest prevalence and the 20-30 ppb interval had frequency 0. Table 9 and Figure 4 show the frequency of isolates based on DON concentration in 0-10 ppb intervals. Our results showed that the 0-3 ppb interval with a frequency 68 (95.8%) had the highest prevalence followed by 5-10 ppb interval; 2 (2.8%) and 3-5 ppb interval; 1 (1.4%) respectively.

Table 10 shows frequency of DON concentration in 0-100 ppb intervals based on species. The Chi-Square test was used to study frequency of DON concentration in 0-100 ppb intervals based on species. Our results showed that there was significant correlation between species and frequency of DON concentration in 0-100 ppb intervals as show in table 11

Table 1: Showing the Chi-Square test to study the effect of geographic criteria and distribution on frequency of species

Chi-Square Tests

	Value	Df	Asymp. Sig. (2sided)
Pearson Chi-Square	55.346	46	0.163
Likelihood Ratio	57.489	46	0.119
N of Valid Cases	107		

Table 2: Showing deoxynivalenol (DON) production mean in culture medium based on species

Species	Count	DON Mean (ppb)
A. af flavus	1	19.228
A. alliaceus	2	35.053
A. awamori	3	5.267
A. candidus	4	28.701
A. carbonarius	6	0
A. flavus	17	10.28
A. foetidus	4	0
A. melleus	3	82.581
A. niger	4	0
A. ochraceus	4	0
A. ostianus	3	1.552
A. parasiticus	5	46.426
A. sojae	8	54.661
A. wentii	3	21.5
A. fumigatus	5	9.935
A. af nidulans	2	4.807
A. niveus	3	35.465
A. terreus	7	1.001
A. unguis	4	7.007
S. ornata	6	56.777
A. spp. III	7	12.603
A. spp. IV	2	6.899
A. spp. V	2	0
A. spp. VI	2	50.803

Table 3: Showing ANOVA test to study the effect of species on deoxynivalenol (DON) production mean

ANOVA Test					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	94760.837	24	3948.368	5.687	0.000
Species	94760.837	24	3948.368	5.687	0.000
Error	57626.169	83	694.291		
Total	152387.006	107			

Table 4: Showing the brief introductive toxin producer isolated species based on deoxynivalenol (DON) acceptable maximum value and Feed & Food reliable international standards

Species	Count of	DON mean	Limits/Std. %

	Isolates	(ppb)	Feed (5000 ppb)	Food	
				Infant (0.0015 ppb)	Adult (1000 ppb)
A. af flavus	1	19.228	0.38	1281866.7	1.9
A. alliaceus	2	35.053	0.7	2336866.7	3.51
A. awamori	3	5.267	0.11	351133.3	0.53
A. candidus	4	28.701	0.57	1913400	2.87
A. carbonarius	6	0	0	0	0
A. flavus	17	10.28	0.21	685333.3	1.03
A. foetidus	4	0	0	0	0
A. melleus	3	82.581	1.65	5505400	8.26
A. niger	4	0	0	0	0
A. ochraceus	4	0	0	0	0
A. ostianus	3	1.552	0.03	103466.7	0.16
A. parasiticus	5	46.426	0.93	3095066.7	4.64
A. sojae	8	54.661	1.09	3644066.7	5.47
A. wentii	3	21.5	0.43	1433333.3	2.15
A. fumigatus	5	9.935	0.2	662333.3	0.99
A. af nidulans	2	4.807	0.1	320466.7	0.48
A. niveus	3	35.465	0.71	2364333.3	3.55
A. terreus	7	1.001	0.02	66733.3	0.1
A. unguis	4	7.007	0.14	467133.3	0.7
S. ornata	6	56.777	1.14	3785133.3	5.68
A. spp. III	7	12.603	0.25	840200	1.26
A. spp. IV	2	6.899	0.14	459933.3	0.69
A. spp. V	2	0	0	0	0
A. spp. VI	2	50.803	1.02	3386866.7	5.08

Table 5: Showing the brief introductive toxin producer isolated species based on deoxynivalenol (DON) acceptable maximum value and Feed & Food reliable international standards

Species	Count of Isolates	DON mean (ppb)	Limits/Std. %		
			Feed (5000 ppb)	Food	
				Infant (0.0015 ppb)	Adult (1000 ppb)
A. af flavus	1	19.228	0.38456	1281866.667	1.9228
A. alliaceus	2	35.053	0.70106	2336866.667	3.5053
A. awamori	3	5.267	0.10534	351133.3333	0.5267
A. candidus	4	28.701	0.57402	1913400	2.8701

A. carbonarius	6	0	0	0	0
A. flavus	17	10.28	0.2056	685333.3333	1.028
A. foetidus	4	0	0	0	0
A. melleus	3	82.581	1.65162	5505400	8.2581
A. niger	4	0	0	0	0
A. ochraceus	4	0	0	0	0
A. ostianus	3	1.552	0.03104	103466.6667	0.1552
A. parasiticus	5	46.426	0.92852	3095066.667	4.6426
A. sojae	8	54.661	1.09322	3644066.667	5.4661
A. wentii	3	21.5	0.43	1433333.333	2.15
A. fumigatus	5	9.935	0.1987	662333.3333	0.9935
A. af *nidulans*	2	4.807	0.09614	320466.6667	0.4807
A. niveus	3	35.465	0.7093	2364333.333	3.5465
A. terreus	7	1.001	0.02002	66733.33333	0.1001
A. unguis	4	7.007	0.14014	467133.3333	0.7007
S. ornata	6	56.777	1.13554	3785133.333	5.6777
A. spp. III	7	12.603	0.25206	840200	1.2603
A. spp. IV	2	6.899	0.13798	459933.3333	0.6899
A. spp. V	2	0	0	0	0
A. spp. VI	2	50.803	1.01606	3386866.667	5.0803

DISCUSSION

Based on our results there was significant statistical differences between obtained *Aspergillus* spp. and deoxynivalenol (DON) production. In our obtained data, the value of measured toxin by competitive direct ELISA showed that all of the mentioned species had toxin production mean value less than permissive limit by FDA (5 ppm or 5000 ppb) for feed (Table 4) (Whitlow LW and Hagler Jr. WM, 1994). However, they had toxin production mean value more than permissive limit by ECS (0.0015 ppb) for infant's food that should not be neglected (Table 4) (Al-Hazmi NA, 2011). They had also toxin production mean value less than permissive limit by FAO (1 ppm or 1000 ppb) for adult's food (Table 4) (Al-Hazmi NA, 2011).

Value of measured toxin by competitive direct ELISA showed that all of the mentioned species had toxin production mean value less than permissive limit by FDA (5 ppm or 5000 ppb) for feed (Table 5) (Whitlow LW and Hagler Jr. WM, 1994). However, they had toxin production mean value more than permissive limit by ECS (0.0015 ppb) for infant's food that should not be neglected (Table 5) (Al-Hazmi NA, 2011). They had also toxin production mean value less than permissive limit by FAO (1 ppm or 1000 ppb) for adult's food (Table 5) (FAO/WHO, 2012). Our data showed that in plate areas, *Aspergillus sojae* with a mean value 60.625 ppb, *A. niveus*

(35.465 ppb) and *A. flavus* (29.128 ppb) had the highest DON production mean value in culture medium. However, in forest areas, *S. ornate* (61.390 ppb), *A. parasiticus* (35.695 ppb) and *A. alliaceus* (35.053 ppb) had the highest DON production mean value and in mountain areas, *A. unguis* (14.013 ppb) had the highest DON production mean value. Also in industrial areas, *S. ornate* (73.142 ppb) and *A. flavus* (21.846 ppb) had the highest DON production mean value. Value of measured toxin by competitive direct ELISA showed that all of the mentioned species had toxin production mean value less than permissive limit by FDA (5 ppm or 5000 ppb) for feed (Table 6) (Whitlow LW and Hagler Jr. WM, 1994). However, they had toxin production mean value more than permissive limit by ECS (0.0015 ppb) for infant's food that should not be neglected (Table 6) (Al-Hazmi NA, 2011).

They had also toxin production mean value less than permissive limit by FAO (1 ppm or 1000 ppb) for adult's food (Table 6) (FAO/WHO, 2012). Our data showed that 107 *Aspergillus* isolates obtained from processing houses and plants. Among them A. *melleus* (82.581 ppb), *A.* spp. VI (50.803 ppb), *A. parasiticus* (49.108 ppb), *A. sojae* (48.696 ppb) and *S. ornate* (48.247) had the highest DON toxin production mean value in culture medium. However *A. candidus* (28.701 ppb), *A.* spp. III (21.342 ppb) and *A.* af *flavus* (19.228 ppb) had moderate toxin production mean value.

Table 6: Showing the brief introductive toxin producer isolated species based on deoxynivalenol (DON) acceptable maximum value and Feed & Food reliable international standards

Species	Count of Isolates	mean DON (ppb)	Limits/Std. %		
				Food	
			Feed (5000 ppb)	Infant (0.0015 ppb)	Adult (1000 ppb)
A. alliaceus	2	35.053	0.70106	2336866.667	3.5053
A. awamori	1	0	0	0	0
A. carbonarius	3	0	0	0	0
A. flavus	11	15.888	0.31776	1059200	1.5888
A. foetidus	1	0	0	0	0
A. niger	1	0	0	0	0
A. ochraceus	2	0	0	0	0
A. ostianus	3	1.552	0.03104	103466.6667	0.1552
A. parasiticus	1	35.695	0.7139	2379666.667	3.5695
A. sojae	4	60.625	1.2125	4041666.667	6.0625
A. wentii	2	32.251	0.64502	2150066.667	3.2251
A. fumigatus	5	9.935	0.1987	662333.3333	0.9935
A. niveus	3	35.465	0.7093	2364333.333	3.5465
A. terreus	3	0	0	0	0
A. unguis	1	14.013	0.28026	934200	1.4013
S. ornata	3	65.308	1.30616	4353866.667	6.5308
A. spp. III	3	0.95	0.019	63333.33333	0.095
A. spp. V	2	0	0	0	0

Value of measured toxin by competitive direct ELISA showed that all of the mentioned species had toxin production mean value less than permissive limit by FDA (5 ppm or 5000 ppb) for feed (Table 7) (Whitlow LW and Hagler Jr. WM, 1994). However, they had toxin production mean value more than permissive limit by ECS (0.0015 ppb) for infant's food that should not be neglected (Table 7) (Al-Hazmi NA, 2011). They also had toxin production mean value less than permissive limit by FAO (1 ppm or 1000 ppb) for adult's food (Table 7) (FAO/WHO, 2012).

Table 7: Showing the brief introductive toxin producer isolated species based on deoxynivalenol (DON) acceptable maximum value and Feed & Food reliable international standards

Species	Count of Isolates	DON mean (ppb)	Limits/Std. %		
				Food	
			Feed (5000 ppb)	Infant (0.0015 ppb)	Adult (1000 ppb)
A. af flavus	1	19.228	0.38	1281866.7	1.92
A. awamori	2	7.9	0.16	526666.7	0.79
A. candidus	4	28.701	0.57	1913400	2.87

A. carbonarius	3	0	0	0	0
A. flavus	6	0	0	0	0
A. foetidus	3	0	0	0	0
A. melleus	3	82.581	1.65	5505400	8.26
A. niger	3	0	0	0	0
A. ochraceus	2	0	0	0	0
A. parasiticus	4	49.108	0.98	3273866.7	4.91
A. sojae	4	48.696	0.97	3246400	4.87
A. wentii	1	0	0	0	0
A. af *nidulans*	2	4.807	0.1	320466.7	0.48
A. terreus	4	1.752	0.04	116800	0.18
A. unguis	3	4.671	0.09	311400	0.47
S. ornata	3	48.247	0.96	3216466.7	4.82
A. spp. III	4	21.342	0.43	1422800	2.13
A. spp. IV	2	6.899	0.14	459933.3	0.69
A. spp. VI	2	50.803	1.02	3386866.7	5.08

Our results showed that the 0-10 ppb interval with a frequency 71 (66.4%) had the highest prevalence. The 50-60 and 60-70 ppb interval with a frequency 2 (1.9%) had the lowest prevalence and the 20-30 ppb interval had frequency 0 (Table 8). Our results also showed that the 0-3 ppb interval with a frequency 68 (95.8%) had the highest prevalence followed by 5-10 ppb interval; 2 (2.8%) and 3-5 ppb interval; 1 (1.4%) respectively (Table 9). Our results showed that there was significant correlation between species and frequency of DON concentration in 0-100 ppb intervals as show in Table 11. (Whitlow LW and Hagler Jr. WM, 1994; Al-Hazmi NA, 2011; FAO/WHO, 2012).

Table 8: Showing the frequency of isolates based on deoxynivalenol (DON) concentration in 0-100 ppb intervals

C of Toxin	Frequency	Percent	Cumulative Percent
0-10	71	66.4	66.4
10-20	7	6.5	72.9
20-30	0	0.0	72.9
30-40	4	3.7	76.6
40-50	4	3.7	80.4
50-60	2	1.9	82.2
60-70	2	1.9	84.1
70-80	5	4.7	88.8
80-90	8	7.5	96.3
90-100	4	3.7	100.0
Total	107	100.0	

C; Concentration

Table 9: Showing the frequency of isolates based on deoxynivalenol (DON) concentration in 0-10 ppb intervals

C of Toxin	Frequency	Percent
0-3	68	95.8
3-5	1	1.4

5-10	2	2.8
Total	71	

C; Concentration

Based to the highest frequency of the DON toxin recognition that was obtained from *Fusarium* spp., now we could say this is the first report of DON observation and estimation or measuring in Aspergilli isolates in the world based on the average levels between the minimum 70 ppb (4.7%) and maximum 80-100 ppb (11.2%) detected toxins which were too much more than the international standards and regulations (Table 10) (Whitlow LW and Hagler Jr. WM, 1994; Al-Hazmi NA, 2011; FAO/WHO, 2012). With the results obtained by our study, it is possible for the DON production genes to be active in *Aspergillus* isolates that could be noticed in addition to *Fusarium* ingredients as the second genera of DON production causative agents. Eventually it is noticeable that DON tracer production genes might be introduced by all of fungi isolates in comparison with *Fusarium* while recognized as the specific mentioned toxin faster than other fungi; because there is a possibility that other fungi also have potency of producers that could be transmitted to them during gene transmission phenomenon. Therefore, the genomic study is necessary to validate that DON or any other analog molecules were activated in the conducted toxin producer species or not. Thus, our results indicated that in addition to *Fusarium*, genus *Aspergillus* has a potent ability to produce DON toxin or alike molecules. However, validation of this issue needs further studies, in particular, by using advanced biochemical or genomic molecular techniques.

Table 10: Showing frequency of deoxynivalenol (DON) concentration in 0-100 ppb intervals based on species

Species		C of Toxin										Total
		0-10	10-20	20-30	30-40	40-50	50-60	60-70	70-80	80-90	90-100	
A. af *flavus*	Count	0	1	0	0	0	0	0	0	0	0	1
	Expected Count	0.7	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	1.0
	% within Species	0.0%	100.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%
	% within C/Culture	0.0%	14.3%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.9%
	% of Total	0.0%	0.9%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.9%
A. af *nidulans*	Count	2	0	0	0	0	0	0	0	0	0	2
	Expected Count	1.3	0.1	0.0	0.1	0.1	0.0	0.0	0.1	0.1	0.1	2.0
	% within Species	100.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%
	% within C/Culture	2.8%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	1.9%
	% of Total	1.9%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	1.9%
A. alliaceus	Count	1	0	0	0	0	0	0	1	0	0	2
	Expected Count	1.3	0.1	0.0	0.1	0.1	0.0	0.0	0.1	0.1	0.1	2.0
	% within Species	50.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	50.0%	0.0%	0.0%	100.0%
	% within C/Culture	1.4%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	20.0%	0.0%	0.0%	1.9%
	% of Total	0.9%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.9%	0.0%	0.0%	1.9%
A. awamori	Count	2	1	0	0	0	0	0	0	0	0	3
	Expected Count	2.0	0.2	0.0	0.1	0.1	0.1	0.1	0.1	0.2	0.1	3.0
	% within Species	66.7%	33.3%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%

	% within C/Culture	2.8%	14.3%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	2.8%
	% of Total	1.9%	0.9%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	2.8%
A. candidus	Count	1	1	0	0	1	0	1	0	0	0	4
	Expected Count	2.7	0.3	0.0	0.1	0.1	0.1	0.1	0.2	0.3	0.1	4.0
	% within Species	25.0%	25.0%	0.0%	0.0%	25.0%	0.0%	25.0%	0.0%	0.0%	0.0%	100.0%
	% within C/Culture	1.4%	14.3%	0.0%	0.0%	25.0%	0.0%	50.0%	0.0%	0.0%	0.0%	3.7%
	% of Total	0.9%	0.9%	0.0%	0.0%	0.9%	0.0%	0.9%	0.0%	0.0%	0.0%	3.7%
A. carbonarius	Count	6	0	0	0	0	0	0	0	0	0	6
	Expected Count	4.0	0.4	0.0	0.2	0.2	0.1	0.1	0.3	0.4	0.2	6.0
	% within Species	100.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%
	% within C/Culture	8.5%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	5.6%
	% of Total	5.6%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	5.6%
A. flavus	Count	15	0	0	0	0	0	0	0	2	0	17
	Expected Count	11.3	1.1	0.0	0.6	0.6	0.3	0.3	0.8	1.3	0.6	17.0
	% within Species	88.2%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	11.8%	0.0%	100.0%
	% within C/Culture	21.1%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	25.0%	0.0%	15.9%
	% of Total	14.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	1.9%	0.0%	15.9%
A. foetidus	Count	4	0	0	0	0	0	0	0	0	0	4
	Expected Count	2.7	0.3	0.0	0.1	0.1	0.1	0.1	0.2	0.3	0.1	4.0
	% within Species	100.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%
	% within C/Culture	5.6%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	3.7%
	% of Total	3.7%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	3.7%
A. fumigatus	Count	4	0	0	0	1	0	0	0	0	0	5
	Expected Count	3.3	0.3	0.0	0.2	0.2	0.1	0.1	0.2	0.4	0.2	5.0
	% within Species	80.0%	0.0%	0.0%	0.0%	20.0%	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%
	% within C/Culture	5.6%	0.0%	0.0%	0.0%	25.0%	0.0%	0.0%	0.0%	0.0%	0.0%	4.7%
	% of Total	3.7%	0.0%	0.0%	0.0%	0.9%	0.0%	0.0%	0.0%	0.0%	0.0%	4.7%
A. melleus	Count	0	0	0	0	0	0	0	1	2	0	3
	Expected Count	2.0	0.2	0.0	0.1	0.1	0.1	0.1	0.1	0.2	0.1	3.0
	% within Species	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	33.3%	66.7%	0.0%	100.0%
	% within C/Culture	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	20.0%	25.0%	0.0%	2.8%

	% of Total	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.9%	1.9%	0.0%	2.8%
A. niger	Count	4	0	0	0	0	0	0	0	0	0	4
	Expected Count	2.7	0.3	0.0	0.1	0.1	0.1	0.1	0.2	0.3	0.1	4.0
	% within Species	100.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%
	% within C/Culture	5.6%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	3.7%
	% of Total	3.7%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	3.7%
A. niveus	Count	1	0	0	0	0	2	0	0	0	0	3
	Expected Count	2.0	0.2	0.0	0.1	0.1	0.1	0.1	0.1	0.2	0.1	3.0
	% within Species	33.3%	0.0%	0.0%	0.0%	0.0%	66.7%	0.0%	0.0%	0.0%	0.0%	100.0%
	% within C/Culture	1.4%	0.0%	0.0%	0.0%	0.0%	100.0%	0.0%	0.0%	0.0%	0.0%	2.8%
	% of Total	0.9%	0.0%	0.0%	0.0%	0.0%	1.9%	0.0%	0.0%	0.0%	0.0%	2.8%
A. ochraceus	Count	4	0	0	0	0	0	0	0	0	0	4
	Expected Count	2.7	0.3	0.0	0.1	0.1	0.1	0.1	0.2	0.3	0.1	4.0
	% within Species	100.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%
	% within C/Culture	5.6%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	3.7%
	% of Total	3.7%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	3.7%
A. ostianus	Count	3	0	0	0	0	0	0	0	0	0	3
	Expected Count	2.0	0.2	0.0	0.1	0.1	0.1	0.1	0.1	0.2	0.1	3.0
	% within Species	100.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%
	% within C/Culture	4.2%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	2.8%
	% of Total	2.8%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	2.8%
A. parasiticus	Count	1	0	0	2	0	0	0	0	2	0	5
	Expected Count	3.3	0.3	0.0	0.2	0.2	0.1	0.1	0.2	0.4	0.2	5.0
	% within Species	20.0%	0.0%	0.0%	40.0%	0.0%	0.0%	0.0%	0.0%	40.0%	0.0%	100.0%
	% within C/Culture	1.4%	0.0%	0.0%	50.0%	0.0%	0.0%	0.0%	0.0%	25.0%	0.0%	4.7%
	% of Total	0.9%	0.0%	0.0%	1.9%	0.0%	0.0%	0.0%	0.0%	1.9%	0.0%	4.7%
A. sojae	Count	3	0	0	0	1	0	0	0	0	4	8
	Expected Count	5.3	0.5	0.0	0.3	0.3	0.1	0.1	0.4	0.6	0.3	8.0
	% within Species	37.5%	0.0%	0.0%	0.0%	12.5%	0.0%	0.0%	0.0%	0.0%	50.0%	100.0%
	% within C/Culture	4.2%	0.0%	0.0%	0.0%	25.0%	0.0%	0.0%	0.0%	0.0%	100.0%	7.5%
	% of Total	2.8%	0.0%	0.0%	0.0%	0.9%	0.0%	0.0%	0.0%	0.0%	3.7%	7.5%
A. spp. III	Count	6	0	0	0	0	0	0	0	1	0	7

Species	Statistic											Total
	Expected Count	4.6	0.5	0.0	0.3	0.3	0.1	0.1	0.3	0.5	0.3	7.0
	% within Species	85.7%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	14.3%	0.0%	100.0%
	% within C/Culture	8.5%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	12.5%	0.0%	6.5%
	% of Total	5.6%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.9%	0.0%	6.5%
A. spp. IV	Count	1	1	0	0	0	0	0	0	0	0	2
	Expected Count	1.3	0.1	0.0	0.1	0.1	0.0	0.0	0.1	0.1	0.1	2.0
	% within Species	50.0%	50.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%
	% within C/Culture	1.4%	14.3%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	1.9%
	% of Total	0.9%	0.9%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	1.9%
A. spp. V	Count	2	0	0	0	0	0	0	0	0	0	2
	Expected Count	1.3	0.1	0.0	0.1	0.1	0.0	0.0	0.1	0.1	0.1	2.0
	% within Species	100.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%
	% within C/Culture	2.8%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	1.9%
	% of Total	1.9%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	1.9%
A. spp. VI	Count	0	1	0	0	0	0	0	0	1	0	2
	Expected Count	1.3	0.1	0.0	0.1	0.1	0.0	0.0	0.1	0.1	0.1	2.0
	% within Species	0.0%	50.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	50.0%	0.0%	100.0%
	% within C/Culture	0.0%	14.3%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	12.5%	0.0%	1.9%
	% of Total	0.0%	0.9%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.9%	0.0%	1.9%
	Count	7	0	0	0	0	0	0	0	0	0	7
A. terreus	Expected Count	4.6	0.5	0.0	0.3	0.3	0.1	0.1	0.3	0.5	0.3	7.0
	% within Species	100.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%
	% within C/Culture	9.9%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	6.5%
	% of Total	6.5%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	6.5%
A. unguis	Count	2	2	0	0	0	0	0	0	0	0	4
	Expected Count	2.7	0.3	0.0	0.1	0.1	0.1	0.1	0.2	0.3	0.1	4.0
	% within Species	50.0%	50.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%
	% within C/Culture	2.8%	28.6%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	3.7%
	% of Total	1.9%	1.9%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	3.7%
A. wentii	Count	2	0	0	0	0	0	1	0	0	0	3
	Expected Count	2.0	0.2	0.0	0.1	0.1	0.1	0.1	0.1	0.2	0.1	3.0

	% within Species	66.7%	0.0%	0.0%	0.0%	0.0%	0.0%	33.3%	0.0%	0.0%	0.0%	100.0%
	% within C/Culture	2.8%	0.0%	0.0%	0.0%	0.0%	0.0%	50.0%	0.0%	0.0%	0.0%	2.8%
	% of Total	1.9%	0.0%	0.0%	0.0%	0.0%	0.0%	0.9%	0.0%	0.0%	0.0%	2.8%
S. ornata	Count	0	0	0	2	1	0	0	3	0	0	6
	Expected Count	4.0	0.4	0.0	0.2	0.2	0.1	0.1	0.3	0.4	0.2	6.0
	% within Species	0.0%	0.0%	0.0%	33.3%	16.7%	0.0%	0.0%	50.0%	0.0%	0.0%	100.0%
	% within C/Culture	0.0%	0.0%	0.0%	50.0%	25.0%	0.0%	0.0%	60.0%	0.0%	0.0%	5.6%
	% of Total	0.0%	0.0%	0.0%	1.9%	0.9%	0.0%	0.0%	2.8%	0.0%	0.0%	5.6%
Total	Count	71	7	0	4	4	2	2	5	8	4	107
	Expected Count	71.0	7.0	0.0	4.0	4.0	2.0	2.0	5.0	8.0	4.0	107.0
	% within Species	66.4%	6.5%	0.0%	3.7%	3.7%	1.9%	1.9%	4.7%	7.5%	3.7%	100.0%
	% within C/Culture	100.0%	100.0%	0.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
	% of Total	66.4%	6.5%	0.0%	3.7%	3.7%	1.9%	1.9%	4.7%	7.5%	3.7%	100.0%

C; Concentration

Table 11: Showing Chi-Square test to study the effect of species on frequency of DON concentration in 0-100 ppb intervals

Chi-Square Tests			
	Value	**df**	**Asymp. Sig. (2-sided)**
Pearson Chi-Square	343.754	184	0.000
Likelihood Ratio	177.695	184	0.617
N of Valid Cases	107		

COMPETING INTERESTS

The authors declare they have no competing interests.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: RF LM NSS SRD. Performed the experiments: RF LM ACN SSF Analyzed the data: RF LM ACN SSF SAM. Wrote the paper: RF LM NSS SRD. Revised the paper: RF LM NSS SRD ACN SSF SAM MM NMHM MNMD JH. All authors read and approved the final manuscript to be published.

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