Aflatoxin and Sclerotia Production in Clinical Isolates of *Aspergillus flavus* Group

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Abstract

Backgrounds: To obtain information about clinical isolates of *Aspergillus flavus* group.

Methods: We examined 55 isolates [45 clinical, 10 reference (6 from culture collections, 4 local reference)] for toxicology, growth rates, and morphological and physiological characteristics. Modified Czapek Agar (CZ) and Malt Extract Agar (MEA) were used for observing microscopic morphology and measuring fungal structures. Two additional media, Potato Dextrose Agar (PDA) and a modified Rice Agar (RA), were used to detect fluorescence under UV light. The presence of aflatoxin in culture extracts was confirmed by thin-layer chromatography (TLC).

Results: 66.6% and 55.5% of clinical samples showed different shade of fluorescence on RA and PDA, respectively, after exposure to UV light. Fifteen (33.3%) of the clinical isolates and 3 (30%) of the reference strains produced sclerotia on Czapek Yeast Agar (CYA) at 37° C. Sclerotia formation was promoted at 37°C in comparison with 28°C on CYA medium (*P*< 0.001). Five (11.11%) of the clinical isolates, the Iranian *A. flavus* soil reference strain and *A. parasiticus* ATCC15517 were confirmed to be aflatoxiginc by TLC. From two clinical toxigenic isolates (of 5) which were fluorescence positive on PDA, only one produced fluorescence on RA after exposure to UV light. Moreover sclerotia production was observed in only 3 of 5 toxigenic isolates. Furthermore one isolate from a sinus specimen was identified as *Aspergillus oryzae*. This is believed to be the first report of sinusitis due to *A. oryza*e from Iran.

Conclusion: Some of clinical *A. flavus* isolates could have aflatoxin and sclerotia producing ability, but not necessarily all aflatoxigenic *A. flavus* isolates are capable of producing sclerotia.

Keyword: *Aflatoxin*, Aspergillus flavus, *Fluorescence, sclerotia, TLC*

Introduction

A.flavus can cause every known variant of aspergillosis, in particular pulmonary and other systemic infections in immunocompromised, persons and rare idiopathic systemic infections in apparently immunocompetent patients (1, 2). Comprised with *A.fumigatus*, however, it causes a higher proportion of infections of the sinuses and lower proportion of pulmonary infections (3, 4). It has also caused onychomycosis, otomycosis, keratitis and cutaneus lesions (1, 2).

A.flavus is a cosmopolitan filamentous fungus that is known to occur mostly in soils, but it is also found in plant products, particularly oil-rich seeds and in living plants (5).

Phenotypic variation in *A.flavus* has been well documented. *Aspergillus* section *Flavi*, commonly referred to the *A.flavus* group, includes *A.oryzae*, *A.soja*e, *A.tammarii*, *A.flavus Link*, *A.parasiticus*, *A. aliaceus* and *A.nomius* (1, 5, 6).

Among these species, *A.flavus, A.parasiticus* and *A.nomius* have received major attention due to their ability to produce potent carcinogenic aflatoxins (5, 7). The immune system is affected by these toxins, leading to suppressed cell-mediated immune responses, reduced phagocytosis and depressed production of complement and interferon (8-10). There are many highly specific and sensitive methods for determining aflatoxin concentration in commodities or in culture, such as high performance liquid chromatography (HPLC), en-

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zyme- linked immunoabsorbent assay (ELISA), thin -layer chromatography (TLC) (11-13). However, these methods are relatively expensive and time consuming. Alternative techniques for rapid screening are growth on a suitable media such as PDA, coconut agar and corn followed by visualization under Long wave UV light (365nm) where toxins can be seen as a bright blue-white or green halo around the fungal colony regarding the fungal species (14,15).

The non-aflatoxigenic species *A.oryzae* and *A. sojae* are widely used for the production of food grade amylase and in the fermentation of sake, miso and soy sauce (5). *A.oryzae* isolates tend to have a floccose colony texture, producing abundant aerial mycelia, whereas *A.flavus* tends to produce more yellow-green shades. There is evidence that, *A.flavus* can appear degenerated after prolonged growth in a patient, with colonies appearing deeply floccose and white with a greenish cast due to scattered conidiophores in the aerial mycelium (1).

The conidia of *A.oryzae* are generally larger than those of *A.flavus* and posses a smooth outer wall (6). *A.oryzae* isolates rarely produce sclerotia in culture whereas *A.flavus* isolates often produce them. Unlike *A.flavus* strains, aflatoxins have not been reported to be produced by *A.oryzae* (5, 16).

Almost all available information on *Asprgillus* section *Flavi* is for strains isolated from soil, food and feed commodities, and relatively little is known about clinical isolates.

Therefore the aim of this study was to perform a systematic study on 45 clinical isolates belonging to *Asprgillus* section *Flavi* from Iran to obtain Information on growth rates, morphological, physiological, and toxicological characteristics.

Materials and Methods

Fourty five isolates of *A.flavus* group were recovered from different patients referred to Mycology Unit, School of Public Health, Tehran University of Medical Sciences in Iran from January 2004 to December 2006. Patients comprised 28 males and 17 females, ranging in age from 1 to 59 yr. Twenty three (51.1%) of pa-

tients had paranasal lesions, 14 with nail involvement, five with pulmonary, one with endocard, one with neck subcutaneous and one with disseminated infection. Underlying diseases were blood malignancy (12), sinus surgery (3), bone marrow transplantation (1), but twelve patients were apparently healthy, and there is no information about 17 of the remaining. Infection was confirmed by direct examination, culture and histopathology.

Additional reference strains of *Aspergillus* section *Flavi* obtained from other sources conducted in this study as follow: *A.flavus* NCPF 2008 (National Collection of Pathogenic Fungi), *A.flavus* JCM 2061(Japanese Collection of Microorganisms), *A.flavus* PTCC 5006 (Persian Type Culture Collection), *A.parasiticus* ATCC 15517 (American Type Culture Collection), *A.oryzae* IMI 126842(International Mycological Institute), *A.sojae* IMI 191303. Local Iranian environmental isolates (local references) were also included; there were two aflatoxigenic *A.flavus* strains isolated from soil and pistachio, one non-aflatoxigenic *A.flavus* from soil and one aflatoxigenic *A.parasiticus* from pistachio. These later four strains are Iranian local references.

All organisms were plated on following different culture media: Czapek Yeast Agar (CYA): K_2HPO_4 1g, Czapek Concentrate (CC) 10 ml, Powdered Yeast Extract (PYE) 5g, Sucrose(Su) 30g, Agar (A) 15g, Distilled Water (DW) 1L. Czapek Dox Agar (CZ): CC 10ml, K_2HPO_4 1g, Su 30g, A 17.5g, DW 1L. Czaek Yeast Agar with 20% Su (CY20S): K_2HPO_4 1g, CC 10 ml, PYE 5g, Su 200g, A 15, DW 1L. Malt Extract Agar (MEA): Powdered Malt Extract 20 g, peptone 1g, Glucose 20g, A 20 g, DW 1L. CC included: $NaNO_3$ 30g, KCl 5g, $MgSO_4.7H_2O$ 5g, $FeSO_4.7H_2O$ 0.1g, and $ZnSO_4$. $7H_2O$ 0.1g, $CuSO_4$. $5H_2O$ 0.05g, DW 100ml.

For each isolate, duplicate cultures were performed. Each plate was inoculated at three points, equidistant from the centre and incubated in the dark for 7 d. A series of CYA plates were incubated at 37° C and rest were incubated at 28° C for 7 d.

Preparation of inoculum A medium consisting of 0.05% tween 80 and 0.2% agar (TA) was

used for the preparation of the spore suspension, in order to prevent the colonies from straying on the plates. The numbers of conidia were adjusted to 1×10^4/ml in TA medium. Two µl of suspension containing 20 conidia was later transferred to each different culture media.

All measurements were taken after 7 d of incubation. Colony diameters were measured by ruler from the undersides of the plates. Slide cultures were performed on MEA for microscopic observation. All culture media were examined after 48 h and 7 d. Morphological characteristics such as colour, texture, sclerotia formation and gross appearance were recorded. Mean diameter of colonies grown on different media were determined and frequencies of mean diameter colonies were recorded. Diameter of zero corresponds to entirely inhibition of fungus (Table 1).

Cultivation and observation of fluorescence

All stock cultures were maintained on (SDA) medium. PDA and modified rice medium agar were prepared according to McGinnis (17) and Cutuli et al. (18). Modified Rice Agar was composed of 32 g rice, 8 g agar in 1 litre DW, pH 6. Slants were used for observing fluorescence. Equal numbers of 20 conidia were inoculated on the centre of two series of slants and were incubated in dark for 10 d. The reverse side of cultures were periodically examined under long-wave UV (365nm) for fluorescence. An un-inoculated tube was observed as control. All cultures were checked after 48 h, 72 h, 7 and 10 d.

Cultivation on Sabouraud dextrose agar

Sabouraud dextrose agar (Merck Germany) was prepared according to the procedure mentioned by manufacturer and distributed in plates. A series of SDA were also supplemented with 500 mg/l cycloheximide. Equal numbers of 20 conidia were inoculated on the centre of SDA and SDAC plates. The plates were incubated at 28 °C in dark and diameters of colonies were recorded after 48 h and 7 d.

Microscopic Morphology

Slide cultures were prepared on MEA. Two slides were prepared from every slide culture and were examined under light and digital Olympus Microscopes (BX57 equipped with a DP 12 camera and Olysia Software). Texture of conidia, conidiophore, and shape of vesicle and seriation of phialide were examined under light microscope. Vesicle, conidium diameter, and also the length of conidiophores were measured by digital microscope. Sizes of five structural elements of each of these three structural shapes were measured randomly, and their mean measures were recorded. Mean size of conidia, vesicles and mean length of conidiophores were grouped in three scores; small, medium, large for conidia and vesicle, and also as short, medium, and long for conidiophores. SPSS version 11.5 US programmed for windows was used to analyse the data.

Aflatoxin production and analysis

Production of aflatoxin from each isolate was verified by TLC using the agar plug and chloroform method with some modifications (19, 20). Isolates were inoculated onto PDA and incubated at 28-30° C for 7 d in dark. For the agar plug method, a small piece of colony (0.6 cm) was tested directly using TLC. In the chloroform method, the whole colony from a Petri dish was extracted with chloroform in stomacher for 3 min, filtered and concentrated at 60° C to near dryness and dried using vacuum evaporator (Heidolph WB 2000). The residue was re-suspended in chloroform and spotted in duplicate on TLC 20x20 Cm silica gel plates (E. Merck, Germany), which were developed in chloroform-methanol (98:2). Aflatoxin spots were visualized under UV light at 365 nm and TLC plates were scanned with TLC scanner 3CAMAG. Mixtures of aflatoxin B1and B2 standards (Sigma, USA) were used for comparisons in each run. All experiments were carried out at least two times.

Results

The most common infection due to *A.flavus* group was paranasal sinusitis, with 23(51.1%) cases followed by onychomycosis with 14(31.1%) cases, pulmonary infection with 5(11.1%) cases. There was one case of each of endocarditis, neck, subcutaneous lesions and systemic aspergillosis.

Table 1 shows the diameters of colonies on different media. Totally 36(80%) of clinical isolates

showed a colony diameter range of 30-59mm on SDAC medium at 28° C whereas growth of 6(13.3%) isolates inhibited entirely (diameter of colony; zero) or partially (diameter of colony; up to 9mm) by 500mg/l cycloheximide on SDAC. *A.oryzae* IMI 126842 and *A.flavus* JCM 2061 were entirely inhibited on the same medium.

Microscopic examination by digital Olympus microscope showed only 5(11.1%) and 3(30%) of clinical and reference isolates with a conidium mean diameter range of 2.58-3.11μm and also 3(6.6%) and 3 (30%) of them with a vesicle mean range of 12.64-16.30μm respectively. 34(75.5%) of clinical and 4(40%) of reference strains had a conidiophore mean length range of 43-211μm, respectively .Results obtained by goodness of fit test and are shown in Table2.

Of 23 paranasal isolates of *A.flavus* group, 13 (56.5 %) showed fluorescence (blue3, green10) on PDA and 17(73.9%) showed fluorescence (pink12, blue2 and one each of blue-green, blue-violet and red) on RA, respectively.

Of 14 isolates from infected nails 10(71.4%) were found to produce different shades of fluorescence on PDA (green 8, blue- green 2) and on RA (pink 8, blue 1, red 1).

Only one (20%) of 5 isolates from pulmonary involvement produced green and blue-violet fluorescence on PDA and RA respectively. Of 6 reference strains of *A.flavus*, 3 (50%) produced different shades of fluorescence (blue 2, green 1) on PDA and (blue 1, pink 2) on RA.

Both (100%) *A.parasiticus* strains produced blue-green (1) and green (1) fluorescence on PDA and pink (2) fluorescence on RA. Both of strains produced sclerotia on CYA-37° C whereas sclerotia formation on CZ-28 °C occurred by only one (50%) of those.

It is worthwhile to say that in present study, *A. oryzae* in contrary to *A.sojae* produced green and pink fluorescence on PDA and RA, respectively, but these two later strains did not formed sclerotia (Table3).

Five (11.11%) of clinical isolates (4 form sinusitis, 1 form onychomycosis) and also one Iranian *A.flavus* soil reference strain and *A.parasiticus*

ATCC15517 were confirmed to be aflatoxigenic (B_1 or B_1 & B_2) by TLC. TLC Chromatographs showed that clinical toxigenic *A.flavus* isolates produce only B_1 aflatoxin whereas reference toxigenic *A.flavus* produced B_1 and B_2 aflatoxins. The amount of aflatoxin B_1 produced by clinical isolates was ranged from 7.5 to 9.3 ng/g medium. Interestingly toxic *A.flavus* produced 13.2 ng/g of medium of aflatoxin B_1. From two clinical toxigenic isolates which were fluorescence positive on PDA, only one produced fluorescence on RA after exposure to UV light. Moreover three of five clinical toxigenic *A.flavus* isolates were found to be sclerotia producers. (Fig. 1 shows sclerotia formation in one of clinical isolates).

Of three (6.6%) isolates from patients with sinusitis one identified as *A.oryzae* according to morphologic characteristics and remainder two isolates showed morphologic details similar to both *A.flavus* and *A.oryzae* and it may presents an intermediate strain as *A.flavus/oryzae*. These three isolates were non-aflatoxigenic but sclerotia production was observed in only one of the *A.flavus/oryzae* strains.
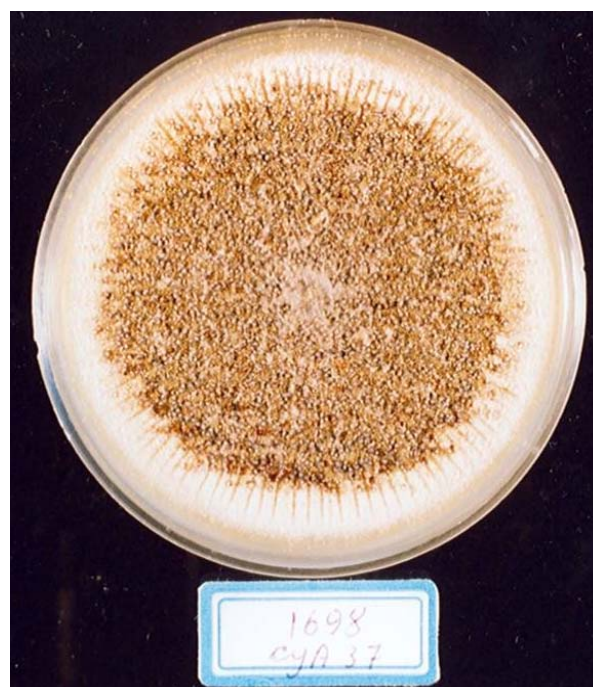

Fig 1: Sclerotia formation in clinical isolate (No.37) after 7 days incubation on Czapek Yeast Agar at 37°C

Table 1: Distribution of clinical isolates of *A. flavus* group on different media according to colony diameter after 7 days at either 28° C or 37° C

Diameters (mm)	Type of Media and temperature											
	CYA[1]-37 °C		CYA[2]-28 °C		CY20-s[3]28 °C		CZ[4]-28 °C		SDA[5]-28°C		SDAC[6]-28°C	
	n	%	n	%	n	%	n	%	n	%	n	%
0-9	0	0	0	0	0	0	0	0	0	0	6	13.3
10-19	0	0	0	0	0	0	0	0	0	0	0	0
20-29	0	0	0	0	1	2.2	1	2.2	0	0	1	2.2
30-39	0	0	0	0	0	0	3	6.6	0	0	9	20
40-49	1	2.2	0	0	0	0	24	53.3	1	2.2	9	20
50-59	4	8.8	4	8.8	2	4.4	16	35.5	2	4.4	18	40
60-65	19	42.2	5	11.1	3	6.6	1	2.2	4	8.8	2	4.4
66-70	21	46.6	36	80.0	39	86.6	0	0	38	84.4	0	0
Total	45	100	45	100	45	100	45	100	45	100	45	100

Note: 1: Czapek yeast agar at 37° C, 2: Czapek yeast agar at 28° C, 3: Czapek yeast agar with 20% sucrose at 28° C 4: Czapek Dox Agar at 28° C, 5:Sabouraud dextrose agar at 28° C, 6:SDA supplemented with 500mg/l Cycloheximide at 28 °C

As shown entirely or partially inhibition of growth clinical isolates of *A. flavus* group occurred on SDAC-28°C

Table 2: Some major microscopic characteristics of clinical isolates and reference strains of *Aspergillus flavus* group on MEA medium at 28°C

Structures	Observed mean size rang(µm)	Type of Group			
		Clinical		Reference	
		n(45)	%	n(10)	%
Conidia diameter(µm)	S:1.7-213 M:2.14-2.57 L:2.58-3.11	23 17 5 χ^2=11	51.1 37.7 11.1 df=2	4 3 3	40 30 30
Vesicle diameter(µm)	S:5.30-8.96 M:8.97-12.63 L:12.64-16.30	25 17 3 χ^2=16.4	55.5 37.7 6.6 df=2	4 3 3	40 30 3
Candiophroe length (µm)	Sh:43-211 M: 212-380 L_1:381-548	34 11 0 χ^2=40.1	75.5 24.4 0 df=2	4 5 1	40 50 10

Note: S: small, M: medium, L: large, Sh: short, L_1= long
Three various mean size range were obtained for each of conidia, vesicles, and conidiophores which are not uniformly distributed.

Table 3: Fluorescence and sclerotia production pattern by clinical isolates of *A. flavus* group in comparison with reference strains on different media

Type of Group	Status of clinical & reference	n. of tested		Fluorescent production & Type of media				Sclerotia formation & type of media							
				PDA[1]		RA[2]		CYA[3]-37		CYA[4]-28		CZ[5]-28		CY20S[6]-28	
		+	ratio (%)	+	Ratio (%)	+	Ratio (%)	+	Ratio (%)	+	Ratio (%)	+	Ratio (%)	+	Ratio (%)
Clinical	Sinusitis	23	51.5	13	56.5	17	73.9	6	26	3	13	3	13	1	4.3
	Onychomycosis	14	31.1	10	71.4	10	71.4	7	50	2	14.2	1	7.1	1	7.1
	Pulmonary infection	5	11.1	1	20	1	20	2	40	0	0	0	0	0	0
	Other infections	3	6.6	1	33.3	2	66.6	0	0	0	0	0	0	0	0
	total	45	100	25	55.5	30	66.6	15	33.3	5	11.1	4	8.8	2	4.4
Reference	*A.flavus*	6	60	3	50	3	50	1	16.6	0	1	16.6	16.6	1	16.6
	A.parasiticus	2	20	2	100	2	100	2	100	1	50	1	50	1	50
	A oryzae	1	10	1	100	1	100	0	0	0	0	0	0	0	0
	A sojae	1	10	0	0	0	0	0	0	0	0	0	0	0	0
	Total	10	100	6	60	6	60	3	30	2	20	2	20	2	20

Note: +: No. of Positive, 1: Potato Dextrose agar, 2: Rice agar, 3: Czapek yeast agar at 37° C, 4: Czapek yeast agar at 28° C, 5: Czapek Dox Agar at 28° C, 6: Czapek yeast agar with 20% sucrose at 28° C

A MCNemar test showed sclerotia formation promoted significantly at 37° C than in 28° C on CYA medium in all 55(clinical and reference) isolates (*P*<0.001).

Discussion

A.flavus is world wide in distribution and has been particularly prevalent in the air of tropical countries. Climate conditions markedly influence the prevalence of *A.flavus* in outdoor air and construction and renovation activities and contaminated of drinking water influence the prevalence of this fungus in home and hospital air (21). Regarding to literature review aspergillosis in Iran, *A.flavus* has been the main fungus isolated from non- dermatophytic onychomycosis in 1989 and recent under publication report as well (22). Moreover, it has been shown by our laboratory data that *A.flavus* was the most common isolated fungal agents from patients with sinusitis during last two years. We believe the prevalence of aspergillosis by this fungus could be due to the high amount of its conidia in our environment and it seems to be important to know if all of our clinical isolates belonging to *A. flavus* or to other strains of *A.flavus* group.

In two studies from Iran, *A.flavus* was the most prevalent *Aspergillus* species to be recovered from the air of hospital wards and homes (23, 24). Therefore this investigation was designed to have a systematic study on 45 clinical isolates belonging to *Asprgillus* section *Flavi* from Iran. Information on growth rates, morphological and physiological characteristics, and toxicological data were obtained.

About half of the *A.flavus* isolates form aflatoxins, very potent carcinogens (25-27). Aflatoxin is the most economically and medically important mycotoxin in the world.

Instability for aflatoxin synthesis by this section has been observed among laboratory isolates from specific strains (28). Such instability has been difficult to document or quantify and has made studies on the biosynthesis and genetics of aflatoxin production difficult to conduct. Production of aflatoxin by toxigenic *Aspergillus* is affected by environmental factors, such as temperature, pH and certain metal salts (29, 30).

The fluorescent detection of aflatoxin under UV light has been variously considered in screening of these compounds (14, 15).

It has been long noted that B and G aflatoxins differ in their ring structures and have different properties in TLC, fluorescing blue -green and green, respectively.

Magnolia et al. (31) by using 88 g/l rice as culture medium for TLC method after one week of incubation period showed that 47% of *A.flavus* and 50 % of *A.parasiticus* produced aflatoxin. However in the present study when 32g/l rice was used, 66.6 % of clinical isolates of *A.flavus* were capable of producing fluorescence, but only 55.5% of those able to fluoresce on PDA. Also in a study on peanut seeds in Argentina all of 185 but 11(5.9%) isolates of *A.parasiticus* showed aflatoxin production by HPLC method, whereas 57% of isolates produced sclerotia on CZ medium supplemented with 0.5% sodium nitrate and 0.5% sucrose as sclerotia promoter at 30° C (32). In contrary to Argentinean investigators, we used CYA and CZ medium, containing 3% sucrose and 0.3% sodium nitrate at 28° C and 37° C, which resulted the same finding at 37° C. Therefore it seems that temperature (37 °C) might have an important role than the rate of sucrose and sodium nitrate.

Sclerotia production was observed at 28 °C and 37 °C by 7 and 18 isolates amongst 55, respectively. This is in agreement with earlier investigation done by Razzaghi which showed only 4 out of 66 isolates from corn field soils were able to produce sclerotia (33). Of 45 clinical isolates, 30 (66.6) produced different shade of fluorescence; pink 22(48.8%), red 2(4.4%) blue 3(6.6%), blue-violet 2(4.4%), blue-green 1(2.2%), fluo-

rescence on RA at 28 °C whereas different shades of fluorescence in 25(55.5%) of clinical isolates were observed on PDA at 28 °C as follow: green 20 (44.4%), blue3(6.6%), blue-green 2(4.4%). The results of this study showed that the green or blue-green fluorescence on PDA could be equal to pink or pink-red fluorescence on RA medium. This is believed to be the first report of pink and pink-red fluorescence on RA.

All two reference *A.parasiticus* strains produced blue- green and green on PDA and pink on RA and both of them produced sclerotia on CAY-37° C but one on CZ-28 °C. Among them, *A.parasiticus* ATCC15517 was aflatoxin producer. Although 5(11.11%) of the 45 clinical isolates were found to be aflatoxigenic but only 3(6.7%) among them were able to produce sclerotia in the present study. In a study on *A.flavus* isolates, 8(26%) from a total of 30 clinical isolates and 12(40%) out of 30 environmental isolates were produced aflatoxin (34). These findings are quite in agreement with reports of Wang et al. (35), Chang et al. (36) and Razzaghi et al. (33).

One isolate from a patient with sinusitis was identified as *A.oryzae* and two other isolates had morphologic details similar to both *A.flavus* and oryzae (*A.flavus/oryzae*).

Review of the literature reveals a limited number of reported cases of infection caused by *A.oryzae* (1, 3, 21) but this is the first report of sinusitis aspergillosis due to *Aspergillus oryzae* from Iran. In the present study we could not identify either *A.parasiticus* or *A.sojae* from clinical isolates by morphological methods.

Finally cycloheximide tolerance test performed to determine whether the clinical isolates could be the pathogenic if those are able to grow in the presence of cycloheximide by use of SDAC (37). The results revealed that 3 isolates from sinus, two isolates from nail and one isolate from pulmonary specimens were sensitive to cycloheximide and remaining 39(86.6%) of clinical isolates were resistant to 500mg/l cycloheximide, while growth of *A.oryzae* IMI 126842, *A.flavus* JCM 2061 were entirely inhibited in the same condition.

Slide culture preparation method used for observing and measurement of structural shape and size, showed smaller mean size than the mean sizes in tease mount preparation (figures are not shown). The similar difference was also seen in the reference strains.

However, no single morphological or physiological method can be used to classify taxa.

In conclusion, evidence from this study indicated that aspergillosis due to *A.flavus* has been increased during past two years in Iran and some of clinical *A.flavus* isolates have aflatoxin and sclerotia producing ability, but not necessarily all aflatoxigenic *A.flavus* isolates are capable of producing sclerotia**.**

Acknowledgements

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