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Original Article

Detection of *Aflr* Gene and Toxigenicity of *Aspergillus flavus* Group Isolated from Patients with Fungal Sinusitis

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Abstract

Background: *Aspergillus flavus* is the second most important *Aspergillus* species causing human infections particularly fungal sinusitis. Since little is known about aflatoxin producing ability of clinical isolates, this study was undertaken to detect the aflatoxigenic isolates amongst these isolates.

Methods: A total of 23 isolates of *A.* spp. which were recovered from patients proved to have fungal sinusitis by morphological and histological methods and also 5 additional aflatoxigenic and non-aflatoxigenic reference of *A. flavus* group strains were studied. The isolates were identified morphologically using Czapek Yeast Agar and *A. flavus* and *parasiticus* Agar (AFPA). Aflatoxin producing ability of the isolates was confirmed by Thin Layer Chromatography. Existing of *aflR* gene the regulatory gene in aflatoxin biosynthesis, were studied in all isolates by PCR method.

Results: All twenty three *Aspergillus* isolates confirmed as *A. flavus* group by their macroscopic and microscopic features. One clinical isolate confirmed as *A. oryzae* by mycological methods. *A. oryzae* as well as *A. flavus* JCM2061 and NCPF2008 and 3 clinical isolates were not able to produce orange pigment on AFPA. From total of 23 isolates 4 (17.4%) confirmed to be aflatoxigenic by TLC method. A banding pattern which matched to *aflR* primers was amplified with approximate size of 800 bp in all 23 clinical *A. flavus* isolates. A larger banding pattern 1050 bp was revealed in clinical isolate; strain no.20 as well.

Conclusion: Some clinical sinus isolates are able to produce aflatoxin and all of studied isolates including; *A. oryzae, A. parasiticus* and *A. sojae* were able to amplify *aflR* gene under our laboratory conditions.

Keywords: Aspergillus flavus, *aflR, Aflatoxigenicity, Rhinosinusitis, AFPA*

Introduction

A. flavus can cause every known forms 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 sinuses and lower proportion of pulmonary infections (1, 2). In certain geographical locations like Saudi Arabia and Sudan with semi arid and arid dry climate, invasive aspergillosis caused by *A. flavus* is more common than that caused by *A. fumigatus* (2- 4).

Asprgillus 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) effect on immune system and causing suppression of cell-mediated immune responses, reduction of phagocytosis and depres-

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sion of complement and interferon production (8, 9).

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). These strains have the aflatoxin biosynthesis cluster gene but it does not appear to be functional (10).

A. oryzae and *A. flavus* are morphologically very similar. *A. oryzae* has been used for centuries in the food fermentation industry and is generally regarded as safe and no strains of *A. oryzae* are known to produce aflatoxin (11).

Phenotypic variation in *A. flavus* has been documented. Raper and Fennell described the *A. flavus* group with nine species and two varieties, mainly based on the colour of the conidial heads and ornamentation of the conidia (12). The section *Flavi* currently contains 18 accepted species (13).

A. oryzae isolates tend to have a floccose colony texture, producing abundant aerial mycelia, whereas *A. flavus* tends to produce more yellow-green shades. The conidia of *A. oryzae* are generally larger than those of *A. flavus* and posses a smooth outer wall (6).

A. flavus and *parasiticus* agar (AFPA) is a selective medium for detecting *A. flavus* and *A. parasiticus* from soil and seeds. This is a rapid method to detect these aflatoxigenic strains from the other contaminant moulds. These strains produce bright yellow-orange pigments by their growth on mentioned media after 3 d (14).

There are many highly specific and sensitive methods for determining aflatoxin concentration in commodities or in culture, such as high performance liquid chromatography (HPLC), ELISA, thin-layer chromatography (TLC) (15-17). However, these methods are relatively expensive and time consuming.

There are at least 23 enzymatic reactions with 15 structurally defined aflatoxin intermediates required for aflatoxin biosynthesis and 29 genes for these enzymes have been cloned (18, 19). The genes encoding the pathway enzymes for aflatoxin biosynthesis are clustered within a 75 kb region of DNA in *A. flavus* (20).

AflR genes play an important role in the aflatoxin biosynthesis pathway by regulating the activity of other structural genes (19, 20). It is important and useful to know if all of our clinical isolates show *A. flavus* characteristics or we can identify any other strains of *A. flavus* group in our clinical isolates. How many of clinical isolates are able to produce aflatoxin? Do all of these isolates have *aflR* gene? This information may contribute to a better understanding of whether there are differences in pathogenicity in this group. In the present investigation we have used a combination of phenotypic and genotypic method to distinguish our clinical isolates which were apparently involved in *A. flavus* group.

Materials and Methods

A total of 23 isolates of *A. spp.* which were recovered from patients confirmed to have fungal sinusitis by mycological and histopathological methods were used in this study. Five standard strains; *A. flavus* NCPF (National Collection of Pathogenic Fungi) 2008, *A. flavus* JCM (Japan Collection of Micro-organisms) 2061, *A. parasiticus* ATCC (American Type Culture Collection) 15517 toxigenic, *A. oryzae* IMI (International Mycological Institute) 126842 and *A. sojae* IMI 191303 were included in this study as reference organisms.

Strains were stored in distilled water (DW) and kept in room temperature for further use. All organisms were plated on following different culture media for morphological studies; 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. 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 (9).

Aspergillus flavus and *parasiticus* agar (AFPA): peptone 10g, YE 20g, ammonium ferric citrate

0.5g, chloramphenicol 0.2g, agar 15g, dichloran 1 ml (18).

Cultivation on CYA and AFPA

All strains were subcultured from DW, onto CYA medium. 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 of TA medium. Two µl of suspension containing 20 conidia was later transferred to each different culture media.

Two µl of inoculum suspension containing 20 conidia were inoculated on the three points equidistant of plates containing CYA and AFPA. The cultures were incubated at 25º C and 30º C after 96 h for 3-7 d. Morphological characteristics of the colonies on CYA were studied. Producing of orange pigment on AFPA was examined.

Aflatoxin production and analysis

Production of aflatoxin from each isolate was verified by TLC using the agar plug and chloroform method with some modifications (16, 17). Isolates were inoculated onto PDA and incubated at 28-30º C for 7 d in dark. For the agar plug method, a small piece of colony (0.6 cm) was tested after treatment with chloroform 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 60º 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 B1 and B2 standards (Sigma, USA) were used for comparisons in each run. All experiments were carried out at least two times.

Preparation of fungal genomic DNA

High- molecular-weight DNA from isolates was prepared as described previously (21). Briefly, the harvested mycelial mass was flash-frozen in liquid nitrogen and ground to a fine powder. The mycelial powder was then suspended to DNA extraction buffer containing: 50 M Tris-HCL, (pH 8.0), 50 mM EDTA, 3%SDS, 1% ß-mercaptoethanol and 50µl of Proteinase-K (20 mg/ml). The suspension was then incubated at 65º C for 1 h and the cellular debris was removed by centrifugation at 3000×g for 15 min. After addition of 25 µl RNase H (10 mg/ml), the suspension was incubated at 37º C for 30 min, extracted once with phenol-chloroform-isomyl alcohol (25:24:1) and once with chloroform-isomyl alcohol (24:1). The DNA was precipitated by addition of an equal volume of ethanol and 3 M natrium acetate, followed by centrifugation at 12000×g for 30 min. The DNA pellet was rinsed with 70% ethanol and resuspended in distilled water.

PCR of* aflR *gene All 23 sinus isolates were tested for amplification of *aflR* gene. The oligonucleotide primers in the PCR were:

5´-AACCGCATCCACAATCTCAT-3´and 5´-GGTGCAGTTCGCTCAGAACA-3´ The prim- - ers were purchased from alfa DNA (Otava, Canada). PCR analysis was carried out with a DNA thermal cycler (Primus, MWG, Germany). Amplification was performed in 50 µl reaction mixture containing 50mM KCL, 10mM Tris-HCL (pH 8.0), 1.5 mM MgCL2, each deoxynucleotide Triphosphate at a concentration of 200µM, 10 pmol of each primer and 2 units of ampliTaq DNA polymerase.

Approximately 0.2 ng of each genomic DNA was used in each PCR reaction. PCR mixture were heated at 94º C for 5 min and then subjected to 30 cycles consisting of denaturation at 94º C for 30s, annealing at 50º C for 1.30 and extention at 72º C for 1.40 min. A final 10- min extention step at 72º C was also included. Fifteen µl of each PCR product was electrophoresed on 1% agarose (Merck Germany) in 1X Tris–Acetic Acid–EDTA buffer and stained with 0.5µg ethidium bromide ml^{-1}. The PCR products were visualised with UV under UV transilluminator after 45 min running the gel and compared with a standard DNA size marker (Biolab, Germany).

Results

Among the total of 23 patients with rhinosinusitis, history of leukaemia, and previous sinus or nasal surgeries were observed in 14 (60.9%) and 9(39.1%) of them, respectively.

All twenty three *Aspergillus* isolates confirmed to belong to *Aspergillus flavus group* by their macroscopic and microscopic features when plated on CYA. None of these isolates resembled neither *A. parasiticus* nor *A. sojae* morphologically, but one of these isolate (No.26) was similar to *A. oryzae morphologically (*Fig. 1).

Reverse of 20 isolates presented orange pigment on AFPA after 96 h and 7 d at 30º C. (Fig. 2). Three clinical isolates [26, 36, 48] failed to show pigment on their reverse colonies (Table 1). From a total of 28 isolates, five (17.8%) showed to be aflatoxin producers by TLC method (Table 1). Using PCR analysis, a banding pattern which matched to *aflR* primers in this study have been amplified with approximate size of 800 bp in all 28 *A. flavus* studied isolates. An unusual larger banding pattern with approximate size of 1050 has also been revealed in one of those isolates (isolate no.20) (Fig. 3).

Fig. 1: Colonies of *Aspergillus oryzae* (isolate no.26) isolated from sinus specimen on czapek yeast agar medium at 25 º C after 7 days.

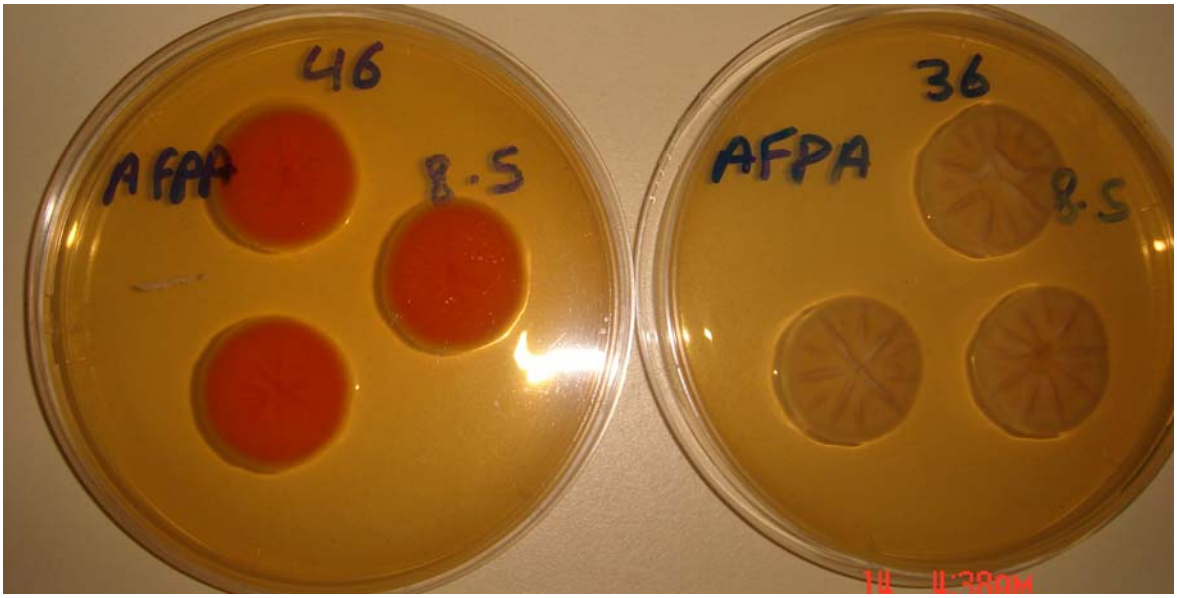

Fig. 2: Reverse of the colonies of clinical *Aspergillus flavus* isolates on *Aspergillus flavus* and *parasiticus* Agar at 30° C after 96 h. Note: the orange and colourless pigmented reverses.

Table 1: Aflatoxin and pigment production pattern of clinical and reference isolates

No of isolates	Isolates	TLC (ng/g)[1]	AFPA[2]
1	reference (*A. oryza* IMI 126842)	negative	orange
4	reference (*A. sojae* IMI 191303)	negative	orange
6	reference (*A. parasiticus*ATCC15517)	12.5	orange
7	reference (*A. flavus* NCPF2008)	negative	colourless
9	reference (*A. flavus* JCM 2061)	negative	creamy
20	clinical (*A. flavus*)	8.1	orange
26	clinical (*A. oryzae*)	negative	colourless
32	clinical (*A. flavus*)	7.5	orange
36	clinical (*A. flavus*)	negative	colourless
40	clinical (*A. flavus*)	9.1	orange
48	clinical (*A. flavus*)	negative	colourless
Others*	clinical (*A. flavus*)	negative	orange

Note: 1: Thin Layer Chromatography, 2: *Aspergillus flavus* and *parasiticus* agar.
*: Isolates no: 2, 3, 15, 16, 17, 22, 23, 25, 27, 34, 33, 38, 39, 41, 44, 46, 57 were the rest of clinical *A. flavus* isolates.

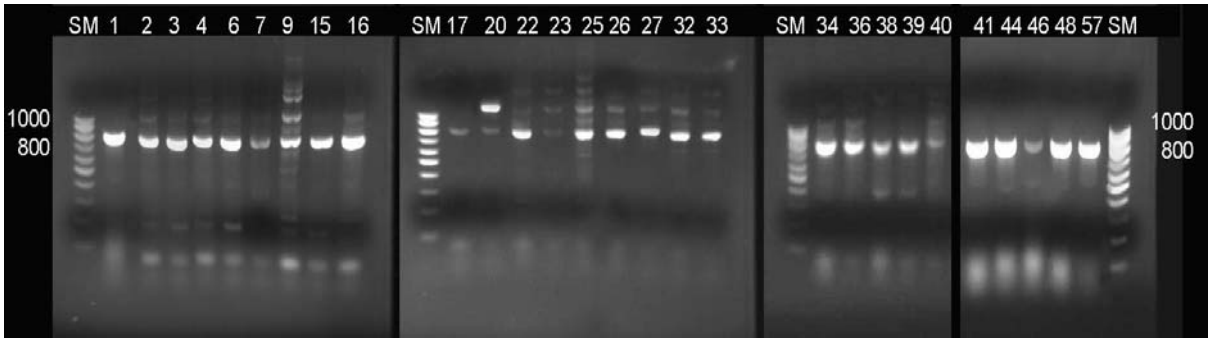

Fig. 3: Amplification of *aflR* gene fragments in 28 isolates of *Aspergillus flavus* group. Isolate no. 20 has shown a large banding pattern with approximate size of 1050 bp.

Discussion

In two studies from Iran, *A. flavus* was the most prevalent *Aspergillus* species to be recovered from the air of hospital wards and homes (22, 23). On the other hand *A. flavus* was found to be the most commonly isolated fungus from nasal polyps in Tehran, Iran (24). Almost all reports of rhinosinusitis come from certain geographical locations (2-4). Whether this reflects climatic conditions, culture and manner of living of residents of these regions or genetic predisposition of them, is unknown. There is a positive correlation between occurring infection and underlying disease and also inoculation of fungus following surgery or other trauma in patients (1, 4). There are rare reports of being *A. oryzae* as the etiologic agent of paranasal sinusitis (4).

One of our studied isolates was identified as *A. oryzae* based on morphological characteristics. The infection was occurred in a 23 yr old male patient following sinus surgery. Up to now there is no documented data regarding *A. parasiticus* or *A. sojae* characteristics being as causative agents of fungal infections (4). None of 22 reminder isolates showed of *A. parasiticus* or *A. sojae* characteristics by morphological methods. There is evidence, morphology of clinical isolates usually varies and may appear as degenerated hyphae after prolonged growth in a patient's tissue therefore colonies appearing deeply floccose with a greenish cast due to scattered conidiophores in the aerial mycelium(1).

A. flavus and *A. parasiticus are* two species produce a distinctive bright orange yellow reverse colour on AFPA after 42-48 h of incubation at 30º C (14).

From a total of 28 studied strains of *A. flavus* group 5 strains (17.8 %) produced colourless or buff reverse on AFPA. The reason why some of our strains were non-orange colour producer is not clear yet, this may be due to lack of ability of the isolates to produce aspergillic acid or neo-aspergillic acid, or alteration of their physiology after being exposed to patient's tissue for prolonged period of time. There are some controversies over the pigment production on AFPA medium by *A. oryzae* and *A. nomius* as well: while some investigations considering the cream production by *A. oryzae* on the reverse side the colony, the other report orange pigment production by the same fungus (5, 25). The failure of orange pigment production by *A. nomius* has been reported by Cotty (26).

In a study on mycotoxigenicity of 30 *A. flavus* clinical isolates by TLC method, 7(23%) produced aflatoxin B1 and one (3%) of them produced aflatoxin G1 (27).

In our study only 4 (17.4%) of 23 *A. flavus* isolates showed to be aflatoxin producer by TLC method. Recently 27.5% of 66 *A. flavus* isolates from corn field soils in Iran was reported to be aflatoxigenic (28).

AflR is a positive regulatory gene which is required for transcriptional activation of most, if not all, of the structural genes. (18- 20, 29)

PCR analysis was able to amplify a fragment by using *aflR* primers in all 23 studied isolates as well as in 5 reference strains. This PCR fragment showed to be 796 bp in all of clinical isolates. However the approximate size of such PCR fragment in isolate no. 20 showed to be 1050 bp in at least 3 repeated PCR. Since the mentioned isolate was able to produce aflatoxin B1 which detected by TLC method, the sequence analysis of its related PCR fragment has been chosen for further analysis in near future.

Aspergillus no.26 was morphologically confirmed as *A. oryzae* and PCR was able to amplify a banding pattern by *aflR* primers in this isolate. Some studies have indicated the existence deletions in *aflR* gene in some *A. oryzae* and *A. sojae* strains. Other studies have shown that an aflatoxin gene clusters is present in some strains of *A. oryzae* and *A. sojae* (30, 31). However the *aflR* genes, in two mentioned species are not transcribed (32, 33).

Although all studied *Aspergillus flavus* group isolates could able to amplify *aflR* gene but only 5 (17.8 %) were aflatoxin producer by TLC method. Our finding confirmed that in our study, all of *A. flavus, A. parasiticus, A. sojae* as well

as two *A. oryzae* could amplify *aflR* gene, but existing of this gene is not enough to producing aflatoxin and many other genes and factors in addition to existing of genes, like kind of culture media, pH and other nutrients are interrupted in aflatoxigenicity of these organisms.

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The authors declare that they have no Conflict of Interests.

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