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ORIGINAL ARTICLE

Could aflatoxin B1 production by *Aspergillus flavus* affect the severity of keratitis: an experience in two tertiary health care centers, Egypt

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Abstract

To investigate the expression of AFB1 gene in isolates obtained from corneal scrapping samples from keratitis patients and to correlate the quantity of AFB1 to the severity of keratitis. An observational study was undertaken in Medical Microbiology and Immunology department, Mansoura University, Egypt, over corneal scrapping samples that were cultured aiming to isolate fungal causes of infective keratitis followed by AFB1 gene detection in *Aspergillus flavus* isolates by nested PCR then quantitation of the toxin by TLC. Out of 843 corneal scrapping samples collected from patients with infective keratitis, positive fungal growth was identified in 277 cases (32.9%). *A. flavus* was the commonest fungal agent isolated in 93 cases (33.6%). The AFB1 toxin–encoding gene was detected in 63.4% of *A. flavus* isolates. There was a positive correlation between the quantity of produced AFB1 toxin and the degree of severity of keratitis (*P* value < 0.0001*). *Aspergillus flavus* was the most common cause of fungal keratitis, with the AFB1 toxin–encoding gene detected in more than half of the isolates. A significant correlation between the degree of severity of keratitis and the quantity of produced AFB1 toxin was detected. Therefore, exploring presence or absence of AFB1 toxin is important for the clinicians in their diagnostic assessment and selection of proper treatment choices.

Keywords *A. flavus* · Aflatoxin B1 · Fungal keratitis · Nested PCR · Thin-line chromatography

Abbreviations

A. flavus	*Aspergillus flavus*
AFB1	Aflatoxin B1
Nested PCR	Nested polymerase chain reaction
TLC	Thin-line chromatography

Introduction

One of the most important ophthalmic problems that cause visual disability is mycotic keratitis. Increasing fungal infections in the last 30 years was due to immunodeficiency disorders, misuse and overuse of antibiotics to treat bacterial infections, and the wide dependence on indwelling devices, besides the worldwide spreading of the use of contact lenses [1].

Aspergillus flavus is considered an important etiology of keratitis second to *Aspergillus fumigatus* [2]. *Aspergillus flavus* produces potent mycotoxins; aflatoxins, which can cause acute hepatic damage, liver cirrhosis, and tumors and has teratogenic effects [3]. Aflatoxin synthesis is encoded by a gene cluster of 30 genes [4] that can lead to multiple types of toxins; the most important of which is aflatoxin B1 (AFB1). Eight out of ten *A. flavus* strains isolated from corneal tissue samples obtained from patients with keratitis was found to produce AFB1 as reported by a previous study. However, strains obtained from the environment when tested showed that only four of 10 strains of *A. flavus* produced the toxin [5].

aflR, which is present in the center of the gene cluster, is considered the main regulating gene needed for the transcriptional activation of other structural genes. aflS, which is found adjacent to aflR, is reported to be another divergently transcribed gene essential in regulation of the transcription process [6]. Thus, *A. flavus* strains can be

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categorized as isolates that can synthesize AFB1 and others that cannot. Several environmental factors incuding temperature, pH, and nutritional sources can widely affect aflatoxins production [7].

Seriousness and severity of infections of the corneal tissue mostly relays on the virulence of the infecting microbes and the underlying condition of the cornea [8].

We tried to find a clue about whether aflatoxin B1 produced by *A. flavus* can affect the severity of the keratitis or not in previously published studies, but actually we could not reach an answer. Thus, in this current work, we aimed to detect the expression of AFB1 gene in isolates obtained from samples of corneal scrapping from cases of infective keratitis and to find out if there was a correlation between the quantification of the AFB1 and the degree of severity of the keratitis. The corneal scrapping samples were withdrawn from keratitis patients in two tertiary ophthalmic centers in Mansoura.

Materials and methods

A total of 843 corneal scrapping samples from patients seeking medical advice in outpatient clinics of two ophthalmic tertiary centers in Mansoura, Egypt, were obtained. Samples were collected under complete aseptic precautions using sterile disposable blades. Those patients showing evidence of infective keratitis were enrolled in this study. Eight hundred four samples were obtained from the Mansoura Ophthalmic Hospital and 39 from the University Ophthalmic Center. The samples were received by Medical Microbiology and Immunology department, Mansoura University, Egypt, through a period of 21 months from 1st of January 2017, to 1st of October 2018. Once samples were obtained, they were subjected to mycological processing. The study protocol acquired an approval from our institutional review board (MS.18.11.360) and an informative consent was received from each study participant.

Data regarding the history of enrolled patients was recorded. Exclusion criteria included any case suffered from keratitis due to a cause other than fungal infection.

Fungal keratitis was clinically graded into: mild keratitis, where infiltration was not exceeding the anterior third of the stroma or 3 mm in diameter; moderate, where infiltration involved one-third to two-thirds of the stroma or 3–6 mm in diameter; and severe keratitis, where infiltration was deep into the inner third of the stroma or 6 mm in diameter [9].

The recruited samples were inoculated onto Sabouraud's dextrose agar (SDA) medium and potato dextrose agar (PDA) (Oxoid, England), in addition to CHROM agar (BBL, Paris, France); then, incubation was done in duplicate at 37 °C and 28 °C for a period extending to 3 weeks followed by further identification of resulting isolates.

Identification of fungal isolates

The resulting fungal isolates were identified depending on macroscopic appearance besides growth rate followed by microscopic examination after KOH 10% and lacto-phenol cotton blue-stained wet mounts to detect typical yeast forms or budding yeast and presence or absence of hyphae, pseudohyphae, and spores. After primary isolation and identification, detected *A. flavus* strains were then subcultured onto SDA slants to be incubated at 25–30 °C. The resulting isolates were kept at 4 °C till further analysis.

Detection of aflatoxin B1 encoding genes in isolated *A. flavus* strains using nested PCR

Aspergillus flavus isolates were re-subcultured in Sabouraud's glucose neopeptone for 72 h aiming for DNA extraction from the resulting growth [10]. We centrifuged the resulting growth at 5000*g* for a period of 5 min till pellets were obtained which were put in STES buffer which consist of SDS (1%) and EDTA (0.01 mol/l), in addition to NaCl (0.5 mol/l) and Tris HCl (0.2 mol/l); then, we added glassy beads followed by vortexing for 10 min and cell debris removal by centrifugation.

Then, we added 100 μg of proteinase K with incubation for 15 min at a temperature of 55 °C followed by adding chloroform in an amount of 0.5 volumes. Resulting DNA was finally precipitated by using same amounts of ice-cold isopropyl alcohol; finally, we washed the resulting pellets using 70% ethanol and put them in sterile water [5].

To carry out nested PCR amplification, two steps were performed. For the first step, we used *aflR*-1 and *aflR*-2 primers set that yielded 800 bps. Then, we used the PCR components resulting from the primary step as templates for the following step. The nested primers were *aflR*-1a and *aflR*-2b that yielded 400 bps (Fig. 1) [5, 11]. The set of primers firstly used was: *aflR-1*: AAC CGC ATC CAC AAT CTC AT and *aflR-2*: AGT GCA GTT CGC TCA GAA CA while that of the second step was: *aflR-1a*: GCA CCC TGT CTC CCC TAA CA and *aflR-2b*: ACG ACC ATG CTC AGC AAG TA.

PCR thermal profile used in the first PCR consisted of predenaturation step at 95 °C for 10 min, then 94 °C for 30 s, 50 °C for 45 s, 72 °C for 1.15 min, and ended at 72 °C for 10 min, whereas that for second PCR was 95 °C for 5 min, then 94 °C for 30 s, followed by 50 °C for 1.25 min, 72 °C for 1.40 min, and terminated at 72 °C for 10 min. The reactions were carried out in a total of 35 cycles. The first one yielded

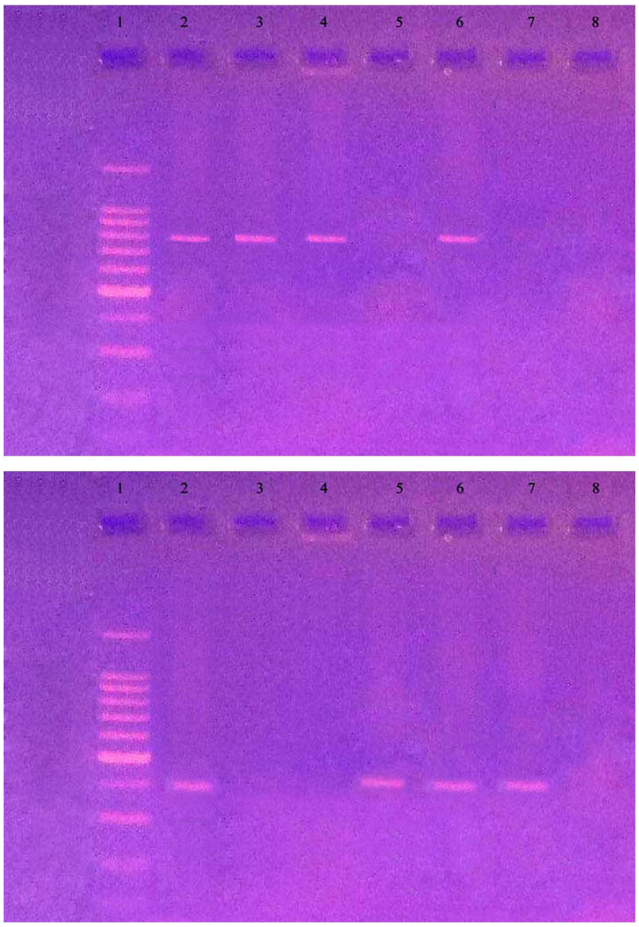

Fig. 1 Gel electrophoresis pattern of first PCR amplification using aflR-1 and aflR-2 primers yielding a DNA 457 fragment of 800 bps (1) and second PCR product using aflR-1a and aflR-2b primers to yield a DNA 458 fragment of 400 bps through a nested PCR amplification (2). Lane 1 showed molecular weight marker 459 #SM0323

800 bps fragments whereas the second reaction yielded 400 bps [5, 12].

Assessment of aflatoxin B1 production by thin-layer chromatography

Aspergillus flavus isolates were re-subcultured in Sabouraud's glucose-neopeptone for 7 days, and the resulting growth was then centrifuged using cooling centrifuge (Heraeus, Hanau, Germany) for 30 min at 4 °C at a velocity of 17,000*g* until a supernatant was obtained. Then, we used a membrane filter with a 0.45-μm pore size (Millipore, Bangalore, India) to filter the supernatant, aiming to remove any source of contamination. The obtained culture filtrate was then used for screening for the production of AFB1 using thin-layer chromatography (TLC) according to the standard method of Przyblylski [12] and Leema et al. [5] modifications. Aflatoxin B1 spots were examined under UV light at 365 nm. The standard AFB1 ((1162-65-8) EEC No 214–603-3, Sigma Aldrich) was used as a control in each run. We enrolled the steps in duplicate.

Quantification of aflatoxin B1 produced by *A. flavus* isolates

It was according to the method reported by Nabney and Nesbitt [13]; first, we scraped off the silica gel with the aflatoxin band away from the chromatography plate; then, it was soaked for 3 min in cold methanol. Second, methanol was removed and the silica gel was washed repeatedly for 5 times with fresh methanol again to bring up the combined filtrate up to 5 ml. We detected the difference between the methanolic filtrate's UV spectrum optical density at 363 nm and at 420 nm to be divided by the extinction coefficient of AFB1, which was 19,800; finally, we multiplied the result by 312 which is the AFB1 molecular weight so as to obtain the final concentration of AFB1.

Statistical analysis

Statistical analysis was performed using the SPSS version 22 (SSPS Inc., PA, USA). Differences between two categorical variables were evaluated using the Chi-square test. Significant differences between three variables were studied using the one-way analysis of variance (ANOVA) with 95% confidence interval (CIs). Pearson's correlation coefficient followed by simple linear regression analysis was applied, to study the correlation between variables. *P* value was significant if less than 0.05.

Results

This observational study was enrolled in Medical Microbiology and Immunology department, Mansoura University, Egypt. It included 843 corneal scrapping samples obtained from patients diagnosed with infective keratitis in Mansoura Ophthalmic centers. On studying the demographic and clinical data of included patients, we found that male patients were more commonly affected than females (523 males versus 320 females), about 158 cases (18.7%) of total keratitis patients were at an age ranging from 11 to 30 years, 409 cases (48.5%) were at 31–50 years, and 220 patients (26.2%) were 50 years old or more (Table 1).

The most commonly reported predisposing factor for keratitis was exposure to trauma (40.6%), followed by topical steroid application (35.1%), then diabetes mellitus (29.1%), usage of contact lens was (23.8%), and lastly, post-ocular surgery-related ulcer was reported in 149 cases (17.7%) only (Table 1). Corneal scrapping samples gave positive mycology

Table 1 Demographic and clinical criteria of study participants

Variable	Number (total = 843)	Percentage (100%)
Gender		
Male	523	62.1%
Female	320	37.9%
Age (years)		
< 10	56	6.6%
11–30	158	18.7%
31–50	409	48.5%
> 50	220	26.2%
Contact lens usage		
Yes	201	23.8%
No	642	76.2%
Past history of any ocular surgeries		
Yes	149	17.7%
No	694	82.3%
Exposure to trauma		
Yes	342	40.6%
No	501	59.4%
History of diabetes		
Yes	245	29.1%
No	598	70.9%
Application of topical steroids		
Yes	296	35.1%
No	547	64.9%

culture results in 277 cases (32.9%) whereas the remaining 566 ones (67.1%) were negative, and these obtained results had a *P* value = 0.001, Table 2.

Aspergillus spp. were the most commonly isolated organism, mainly *A. flavus* in 93 cases (33.6%), *A. niger* in 68 patients (24.6%), *A. fumigatus* in 51 cases (18.4%), and 3 cases (1.1%) in each of *A. terreus* and *A. nidulans* followed by *Alternaria spp.* in 26 cases (9.4%); *Mucor* was isolated from 12 patients (4.3%) and *Fusarium* spp. in 8 (2.9%). *Candida albicans* was reported in 6 cases (2.1%) whereas non-albicans *Candida* was in 5 participants only (1.8%), and the least recorded isolate was dematiaceous fungi in 2 patients only (0.7%) Table 3.

On estimating encoding gene of AFB1 toxin in *A. flavus* isolates using nested PCR, our results revealed that 59 isolates were positive and 34 negative. The results were significant (*P* value = 0.013*) (Table 4). Patients with *A. flavus* AFB1 gene positive were divided according to clinical severity of keratitis into 16 cases (27.1%) with mild keratitis, 14 with moderate condition (23.7%), and 29 patients (49.2%) presented with severe keratitis Table 5**.**

Table 2 Results of the culture of corneal scrapping samples

Participants of the study	Results of corneal scrapping culture on SDA	
	+ve	-ve
Total (*N* = 843/100%)	277 (32.9%)	566 (67.1%)
P value	< 0.0001*	

SDA Sabouraud's dextrose agar

On quantitation of expressed AFB1 toxin produced by isolated *A. flavus* using TLC followed by spectrophotometry and correlating the results to the severity of keratitis, it was noted that the quantity of produced toxin was positively correlated to the degree of severity (*P* value < 0.0001*). The measured mean in mild keratitis cases was 209.08 ± 6.06 ppb, that in moderate cases was 309.42 ± 5.55, and lastly, that of severe keratitis cases was 500.53 ± 53.76 ppb Table 6**.**

We also conducted a simple linear regression model in order to predict the change in severity of fungal keratitis as a dependent variable, and so, the change in quantity of aflatoxin as an independent variable by calculating Pearson's correlation coefficient was 0.949 and one-tailed significance was < 0.0001*.

Discussion

Ocular fungal infections are important causes of serious vision loss and blindness worldwide [14]. In the present study, out of all corneal scrapping samples taken from suspected infectious keratitis, fungal corneal infections represented 32.9%.

Aspergillus keratitis cases were most common among the fungal keratitis. The commonest *Aspergillus* spp. were *A. flavus* (33.6%), *A. niger*, and *A. fumigatus*. This is in comparison to most studies in Egypt [15–17]. This current study reported *Alternaria* spp. in 26 cases (9.4%), *Mucor* in 12 patients (4.3%), and *Fusarium* spp. in 8 (2.9%). *Candida albicans* was also detected in 6 cases (2.1%) whereas non-albicans Candida was in 5 participants only (1.8%). The least recorded isolate was dematiaceous fungi in 2 patients only (0.7%).

Aspergillus keratitis was more prevalent among farmers in geographical regions with predominating hot and humid climates. Trauma by vegetable matter is a main risk factor [18]. We concluded that the most common predisposing factor was trauma (40.6%), topical steroid application (35.1%), diabetes mellitus (29.1%), contact lens (23.8%) and post-ocular surgery-related ulcer (17.7%). These risk factors were different from one study to another and from one country to another, according to different predisposing factors that might help the growth of certain fungi like humidity and temperature [16, 19–22].

Molecular methods based on DNA detection are more sensitive and specific for the detection of *Aspergillus* species in corneal scrapping samples and of aflatoxigenic fungi [23]. In this study, nested PCR was used for detection of the AFB1

Table 3 Types of isolated fungal organisms from corneal scrapping samples culture

Causative agent	Number (total = 277)	Percentage (100%)
Aspergillus flavus	93	33.6%
Aspergillus fumigatus	51	18.4%
Aspergillus niger	68	24.6%
Aspergillus terreus	3	1.1%
Aspergillus nidulans	3	1.1%
Alternaria species	26	9.4%
Candida albicans	6	2.1%
Non-albicans Candida	5	1.8%
Fusarium species	8	2.9%
Mucor	12	4.3%
Unidentified dematiaceous fungi	2	0.7%

gene of *A. flavus* isolates. The results showed that 63.4% was positive for the presence of the gene and 36.6% were negative ones. There was a significant *P* value of 0.013*. In a study by Selvam et al.,47.6% of *A. flavus* was aflatoxogenic strains [24]. The aflR-1 and aflR-2 genes are regulators for structural genes in the aflatoxin gene cluster. The negative PCR may be due to gene deletion from within clusters* and thus the amount of the toxin aflatoxin and the severity of the disease [25].

According to the severity of keratitis, patients with *A. flavus*-positive cultures and ATB1 gene-positive were grouped into mild (27.1%), moderate (23.7%), and severe (49.2%) in order to investigate the relation between the AFB1 production and the severity of the keratitis.

Leema et al. observed higher significant expression levels of aflR in aflatoxigenic than in non-aflatoxigenic *A. flavus* corneal isolates. In addition, a significant positive relation between the expressing of aflR and the mean of aflatoxin production in corneal and environmental isolates was recorded [26]. The study proposed that the expression of structural genes requires special factors to induce transcription [27].

The toxic effects of aflatoxins in the cornea involve corneal haziness, corneal lamellae separation, and infiltration by polymorphonuclear leucocytes [28]. Eighty percent of aflatoxin produced by *A. flavus* was found to be aflatoxin B1 [29].

Table 4 Results of nested PCR detecting aflatoxin B1 genes in obtained *A. flavus* isolates

Nested PCR	Total No of isolates (93/100%)	
	Number	Percentage
Positive	59	63.4%
Negative	34	36.6%
P value	0.013*	

*Statistically significant

Nevertheless, Selvam et al.'s [24] study observed aflatoxin B2, G1, and G2 being more common compared with aflatoxin B1. The higher aflatoxin frequency by the clinical *A. flavus* compared with environmental strains may be a result to selection pressures of different sources mostly antifungal therapy and toxic factors produced from corneal epithelium or immune cells infiltrating diseased corneal tissue [29].

The adaptive value of aflatoxin may play a defensive role against oxidative stress [30]. It was noticed that aflatoxin production has been shown to be reduced by antioxidants [11].

By quantitation of the AFB1 by TLC, the results were correlated with the degree of the severity of the keratitis. It was obvious that there was a strong correlation between the AFB1 quantity and the different groups of the keratitis ($P < 0.0001$*). As declared by Table 6, the measured mean in mild keratitis cases was 209.08 ± 6.06 ppb, that in moderate cases was 309.42 ± 5.55 ppb, and in severe keratitis cases was 500.53 ± 53.76 ppb. TLC method results in comparison with HPLC and ELISA were found to agree between methods but with an advantage of cheapness [31].

It was emphasized that if an *A. flavus* strain isolated from a human lesion is found to be an aflatoxin producer, it is mandatory to treat such fungal lesion not only using the common antifungals, but also we should use molecules that abolish the aflatoxin release or neutralize their deleterious effects [29]. Actually, we are not indifferent about the relation of the aflatoxin production and the infected corneal tissue and if the corneal tissue may be different from the in vitro environment

Table 5 classification of *A. flavus* culture positive cases according to the severity of keratitis

Variable	Number (total = 59)	Percentage (100%)
Mild keratitis	16	27.1%
Moderate keratitis	14	23.7%
Severe keratitis	29	49.2%

Table 6 Correlating the results of quantitation of aflatoxin B1 produced by *A. flavus* isolates to the severity of keratitis

Severity of keratitis	Afatoxin B1 level (ppb)	
	Mean	SD
Mild keratitis	209.08	± 6.06
Moderate keratitis	309.42	± 5.55
Severe keratitis	500.53	± 53.76
P value	< 0.0001*	

*Statistically significant

ppb:parts per billion

especially the toxin, but we correlate the severity of the disease and the presence of the spotted gene and the production of the toxin. We considered that enough as a start point in this field of study.

Activation of regulatory fungal genes such as *aflR* and *aflJ*, which regulate the aflatoxin production, may persist even after the fungus was isolated in culture from corneal scrape material and subcultured one more time to culture media [32].

These study results are needed to be confirmed further by detection of the toxin in the corneal tissues from patients with different corneal keratitis grades, also, to assess the activity of toxin in relation to the severity of the disease.

Conclusion

Aspergillus *flavus* was the commonest etiological agents of fungal keratitis in the studied population with the AFB1 toxin–encoding gene detected in more than half of the isolates. There was a significant correlation between the degree of severity of keratitis and the quantity of produced AFB1 toxin. Therefore, detection of AFB1 toxin is important for the clinicians in their diagnostic assessment. Furthermore, it might guide physicians to use aflatoxin-suppressing molecules in addition to standard antifungals.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The study protocol acquired an approval from our institutional review board (MS.18.11.360).

Informed consent An informative consent was received from each study participant.

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