Molecular profile of aflatoxigenic and non-aflatoxigenic isolates of Aspergillus flavus isolated from stored maize

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ABSTRACT

Maize is a significant staple crop and utilized in Saudi Arabia as food and feed, but maize is often infected with Aspergillus flavus in tropical and subtropical climates, especially during storage. This study intended at a polyphasic approach, consisting of microscopic morphological, biochemical, and molecular characterizations that were applied to 29 of A. flavus isolates of stored maize, with the goal of characterization and identification of aflatoxigenic and non-aflatoxigenic A. flavus isolates. The technique of real-time PCR (RTi-PCR) was used to detection of A. flavus in stored maize samples, the findings have been very accurate.

Centered on macroscopic morphological (primarily colony color and morphology of conidia) and microscopic (morphology of conidia and size) characteristics. Results have shown 23 A. flavus isolates (80%) were categorized as the dark green of colonies also all isolates were rough conidia. The isolates have been two different groups, 16 isolates (62%) had sclerotium-forming and the remaining 13 isolates (38%) had no sclerotium-forming isolates. To the identification of aflatoxigenic isolates of A. flavus in stored maize, we utilized the qualitative methods (easy and inexpensive) like UV test, yellow pigmentation, and ammonia vapor and quantitative method as HPLC (accurate and expensive). the accuracy methods to the identification aflatoxigenicity isolates, vary, and classified in the following descending order: HPLC (100%) > UV method (81%) > yellow pigmentation (YP) and ammonia vapor (AV) (63%). The profile of Aflatoxigenicity of A. flavus isolates by HPLC has been involved in two types first of 11 isolates (38%) have been aflatoxigenic isolates while 18 isolates (62%) were non-aflatoxigenic isolates. The expression of six aflatoxins (AFs) genes (aflD, aflM, aflO, aflP, aflR, and aflQ) was estimated using PCR and RT-PCR. PCR of all genes did not correspond to the aflatoxigenic isolates. The transcriptional analysis of aflO and aflQ was a beneficial marker for discriminating aflatoxigenic from non-aflatoxigenic A. flavus isolates. Also, qRT-PCR indicated that non-aflatoxigenic isolates had a high incidence of defect or downregulation in late AF-genes contrast with early AF-genes, therefore, these non-aflatoxigenic isolates could be critical factors for an efficient and competent strategy for the control of aflatoxin contamination pre-harvest can be considered.

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1. Introduction

Maize (Zea mays L.) is one of the most important cereal crops for human consumption and animal feeds in the world, and Saudi Arabia. Saudi Arabia imported 1.49 million tons valued at $319.23 million in 2016 (Arab Organization for Agricultural Development, 2006). Fungi are normal flora in the maize grains but when conditions favorable for fungal invasion, it constitutes a critical point that may affect the safety, quality, and quantitatively and qualitatively properties of the maize grains (Laca et al., 2006). Fungal invasion of maize grains occurs during crop growth, harvesting, postharvest drying, transportation, and storage, and invasion may be continued which leads to extremely potential fungal risks associated with maize grains (Magan and Aldred, 2006). In fact, maize could be good material for some fungi, especially toxigenic fungi like Aspergillus and Fusarium species (Queiroz et al., 2012). Aspergillus section Flavi is one of the most essential sections in the Aspergillus genus, also these fungi ordinarily contaminated crops.
Aspergillus section Flavi consists of two groups of species but the important one includes A. flavus and A. parasiticus (Cary and Ehrich, 2006; Ehrlich et al., 2007). Another group involving the non-producing aflatoxin species A. oryzae and A. sojae (Kumeda and Asao, 2001), the Taxonomy of Aspergillus section Flavi have been complicated and ever-evolving. Hence, the rigor and reliability of their identity is critical for maintaining an accurate taxonomic system for agricultural, industrial, economic, and regulatory purposes (Pitt and Hocking, 2009). In the past, many researchers have been described Aspergillus section Flavi based primarily on traditional methods based on morphological parameters such as the diameter of a colony, color, and texture, size of conidia and conidiospore structure (Klich, 2002). Classic methods for identifying A. flavus in the laboratory were the time-consuming and laborious for followed up morphological characteristics and get accurate results from professional microbiologists (Lievens and Thomma, 2005). PCR technology has been based on DNA is granted a significant choice for accurate identification of A. flavus with high specific, sensitive, and rapid method (Somasekhar et al., 2004; Godet and Munaut, 2010).

A. flavus remain the most powerful and representative source of naturally occurring aflatoxins (AFs) in the food chain (Cary and Ehrich, 2006). The mycotoxigenic profile A. flavus included many kinds of aflatoxin like AFB1,2 and AFG1,2 have been routinely used for identification aflatoxicogenic or non-aflatoxicogenic isolates. Based on the development of mycotoxin, the classification of A. flavus populations have extremely diverse and included various groups (Vaamonde et al., 2003; Razzaghi-Abyaneh et al., 2006). In the past, molecular-DNA methods have been usually utilized in the differentiating aflatoxicogenic and non-aflatoxicogenic A. flavus isolates through the behaviors of aflatoxin genes (presence or absence) and capacity or incapacity to produce aflatoxins. For example, many PCR systems like Quadruplex and multiplex-PCR were designed for detecting specific genes of AFs (Criseo et al., 2001; Chen et al., 2002).

Recently, the function of genomics (RNA) has developed our knowledge of complicated regulatory machinery, and networks have the mapping of controlling the genes of AFs and their behavior for production AFs. Transcriptomic and proteomic analyses together sharing in knowing the differential expressions of the AFs genes and carbon genes to get the comprehensive model of AFs-genes (Mayer et al., 2003; Rodrigues et al., 2009; Rodríguez and Asao, 2001). The Aim of this research was to characterize 29 A. flavus isolates (focus on non-aflatoxicogenic) recovering from stored maize, in Saudi Arabia, based on a polyphasic approach that involves morphological, biochemical, and molecular methods.

2. Materials and methods

2.1. Fungal isolation and identification

For isolation and identification of seed-borne fungi, thirty corn grain samples, collected from various locations in Riyadh region, were used. The samples were disinfected and put aseptically on an agar petri dish containing Rose Bengal in triplicate and incubated at 25°C (FDA, Difco Laboratories, USA) and the method continues according to (Singh et al., 1991). Isolated fungi were purified either by hyphal tip or single spore methods, and identified according to the methods of (Pitt and Hocking, 1997; Klich, 2002). Designated with the AsFmRnn code, where AsF refers to the Aspergillus flavus genus, M refers to the product (maize), R refers to the area (city of Riyadh) and the isolate number is nn. For subsequent morphological characterization, all isolates were kept on PDA tubes and then kept at 4°C.

2.2. RTi-PCR assay

To help rapid screening for the appearance of A. flavus in stored maize samples, two specific primers were used in one reaction mixture for RTi-PCR. RTi-PCR amplifications were performed with a LightCycler 2.0; (Roche Diagnostics GmbH, Germany) using primer Forward CTCCCACCCGTTTACTGT, Reverse GCGTTTCCTCATC-GATGCCT, according to (Rong et al., 2006).

2.3. Morphological characterization

A loop full of spores in 500 μL of 0.2 percent agar was suspended for each isolate and this suspension was used for three-point inoculations on 9 cm diameter Petri dishes containing 20 mL of MEA and C2. Isolates were incubated at 25°C for 7 days, in the dark, and then examined for colony colour, appearance and scale of sclerotia, and morphology of conids. Identification of isolates by endorsing the available taxonomic keys and guides for the genera of Aspergillus (Klich, 2002; Samson et al., 2004).

2.4. Differentiation of aflatoxicogenic and non aflatoxicogenic A. Flavus isolates

2.4.1. Qualitative methods

2.4.1.1. Ultraviolet light (UV) test and yellow pigmentation. Isolates were grown at 25°C for 7 days on the coconut agar medium. Petri dishes were observed under long UV light (365 nm) after incubation. The presence of a fluorescent zone around the growing fungal colony and blue/violet fluorescence on the reverse side suggested that only aflatoxin B could be generated by an isolate, while a blue/white fluorescence suggested that aflatoxin B and G were generated by an isolate, the absence of fluorescence revealed that an isolate failed to generate detectable values of aflatoxin B and G (Davis et al., 1987; Dyer and McCammon, 1994). We detected orange-yellow pigmentation on the reverse side of the petri dish during the incubation phase, which makes the isolate harmonious with AF production (Lin and Dianese, 1976).

2.4.1.2. Ammonia vapor test. The isolates were grown in the dark on the yeast extract sucrose (YES) media and incubated at 28°C for 3– 4 days. During incubation, the color of the media transforms pink to red during applying a 500ul ammonia solution (Sigma-Aldrich) was seen by aflatoxicogenic isolates. While no color shift for non-aflatoxicogenic isolates was observed (Saito and Machida, 1999).

2.4.2. Quantitative method

2.4.2.1. HPLC analysis. All isolates were grown in the medium of YES were examined for the production of aflatoxins. Isolates were inoculated at 25°C to 6 cm in diameter Petri dish for 7 days and continue the technique of (Bragulat et al., 2001). Samples were analyzed using a HPLC PerkinElmer series 200 UV / VIS unit provided with a UV detector, and fluorescence was measured using wavelengths of 365 nm excitation and 430 nm emission. On the C18 column, chromatographic separations were carried out, HPLC conditions such as the mobile phase, the total run time, the amount of injection were applied as (Christian, 1990). A standard HPLC chromatogram of aflatoxins was plotted, and the concentrate samples were quantified.
3. Results

3.1. Distribution of isolates of Aspergillus section Flavi

The recovered fungal taxa were greatly influenced by various locations of the samples of maize grains and thus LSD was used to compare the degree of isolation frequency for each fungus (Table 2). Isolation frequencies of Aspergillus genera especially A. flavus and Fusarium genera significantly developed when storage maize grains were collected from eastern and southern regions of Riyadh city. These results may indicate that these fungi affecting storage conditions like different temperatures and humidity also, competition or antagonism between various fungal genera. Hence, the findings showed a significant increase or decrease in their isolation frequencies. The results reported the superiority of Aspergillus genera in all the storage maize samples analyzed with the mean of 34% followed by Fusarium genera 22.25% (Fig. 1). The other species isolated belonged to the Aspergillus section Nigri and Terrei colonization of maize grains were lower in stored samples (12% and 4.75%). The relative distribution of fungal isolates over samples explained that A. flavus was the highest distributed 17.25%.

3.2. A. Flavus specific real-time PCR assay

Melting temperature (Tm) values were 92.58°C for 10 A. flavus isolates (Fig. 2). The presence of the one signal of xx only, and not for any other Aspergillus genera, like A. niger and A. terreus, has also confirmed the real-time specificity.

3.3. Morphological analysis

A total of 29 A. flavus isolates were grouped into six separate morphology-based chemotype profiles (primarily sclerotia, colony color, and conidia morphology) Table 3. Chemotype I (n = 6), Chemotype II (n = 7), Chemotype 3 (n = 3), Chemotype IV (n = 7), Chemotype V (n = 2) and Chemotype VI (n = 4). The main A. flavus isolates was sclerotial production (16 isolates 62%) divided to L-type forming (9 isolates, 31%), and S-type forming (4 isolates,13.8%). No sclerotium-forming (13 isolates, 38%). 23 isolates (79.3%) were categorized as the dark green of colonies also all isolates were rough conidia.

3.4. Differentiation of aflatoxicogenic and non aflatoxicogenic isolates

3.4.1. Qualitative methods

Among the 11 isolates, 9 (81%) isolates were categorized as potentially aflatoxicogenic based on blue fluorescence when exposed to UV light in CAM (Table 4). Yellow pigmentation and ammonia vapour test have the same efficiency in identifying aflatoxicogenic isolates (63%), 7 isolates.

3.4.2. Quantitative of aflatoxins by HPLC

29 A. flavus isolates were split into three chemotypes (Table 5). Chemotype I was the large preponderance (62%) were non-aflatoxicogenic isolates followed by 31% of aflatoxicogenic isolates (AB1) belong to chemotype II and finally, chemotype III involved 7% of aflatoxicogenic isolates (B1 + B2). AB1 was produced by eleven isolates (38%) and AB2 was produced by only two isolates Table 6.

Table 1

| Primer code | Gene | Primer sequence (5′–3′) | PCR product size (bp) | RT-PCR product size (bp) |
|-------------|------|------------------------|----------------------|------------------------|
| Nor1-F      | aflD | ACGGATACCTTACCCAGCAC   | 990                  | 815                    |
| Nor1-R      | aflM | CTCAGGCAAGGGAAGGATCCC  | 899                  | 756                    |
| Ver1-F      | aflM | CTTTGATATGAAAAATG     | 999                  | 999                    |
| Ver1-R      | aflR | GCGATGTCGTCGACTCAAA    | 1333                 | 1131                   |
| Afr1-F      | aflO | GCCCGAGATCGAAACACCATC  | 1490                 | 1210                   |
| Afr1-R      | aflP | GCTGCAACAACAACACTCA    | 1088                 | 1088                   |
| Ord1(F)-F   | aflQ | CGGTGTTAGACCGCCAGT    |                      |                        |
| Ord1(F)-R   |     |                        |                      |                        |

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The highest value of B1 97.2 μg/mL was showed by isolate AsF_mR36, and lowest amount of B1 23.2 μg/mL showed by AsF_mR34. Four isolates were very high B1 producers more than 60 μg/mL. All A. flavus isolates displayed a harmonious between HPLC analysis and aflatoxigenic or non-aflatoxigenic profile.

3.5. Molecular analysis with PCR and RT-PCR

The findings of the PCR revealed that six genes, aflD, aflM, aflO, aflP, aflQ and aflR were found in either aflatoxigenic or non-aflatoxigenic isolates and had a non-diverse at PCR model (data not shown). Consequently, to differentiate between aflatoxigenic and non-aflatoxigenic isolates, such a PCR pattern would not be found useful. A. flavus isolates were analyzed by RT-PCR (gene expression), showing a more diverse pattern and relationship to their ability to produce AB1 (Table 6 and Fig. 3). Some gene the expression of the AF-genes was strongly correlated with AF production. We also categorized aflatoxigenic isolates into two types: type I produced AFB1 in amount of ≥ 30 ng/mg fungal dry weight and type II ≤ 30 ng/mg fungal dry weight. AflQ expression was pos-
chemotypes of A. flavus isolates based on mycotoxigenic profile by HPLC. Detection of aflatoxigenic and non-aflatoxigenic isolates of A. flavus. Morphological characterization of isolates of A. flavus. amplifications of aflM (41.4%) and aflP (48.3%) and aflQ (41.4%).

3.6. Gene expression analysis by qRT-PCR

In this analysis, the AF-genes are divided as early pathway genes (aflD and aflR), middle pathway genes (aflM), and late pathway genes (aflO, aflP, aflQ). There was more variation in the expression pattern of the AF-genes. The findings revealed that the defect/deletion was clear in most of the non-aflatoxigenic isolates in the middle and late stages of aflatoxin synthesis genes Fig. 5. In comparison, in most of the genomic DNA of non-aflatoxigenic A. flavus were amplified by the same primers effectively.

4. Discussion

In Saudi Arabia, climatic conditions are very hot and humid and inadequate storage practices provide significant isolation of various seed-borne fungi are associated with maize grains. In our previous study (Mahmoud et al., 2014), A. flavus, Fusarium, Penicillium, and Alternaria species were the major fungal most commonly isolated from maize grains. In the present analysis, isolation of various species of fungi from maize grains could be assigned to (1) long-term preservation of grains in an adequate climate for the occurrence of moldy (2) ideal nutrient composition of maize grains, making it a very strong fungal substratum (3) During the processing of drying maize grains as well as transport between countries, mechanical damage occurred (Mahmoud et al., 2014).

Real-time PCR technology opens up developing opportunities to identify and study phytopathogenic fungi. It combines the sensitivity of traditional PCR with the production of a unique fluorescent signal, providing a real-time study of reaction kinetics and enabling qualitative and quantitative of specific DNA targets (Schena et al., 2004). RT-PCR enables an accurate, reliable, and high specific of target fungal DNA in mycotoxigenic fungi like A. flavus (Mahmoud, 2015), Fusarium (Nicolaisen et al., 2009), and Penicillium (Rodrigues et al., 2011) in food or feed products.

In the present study, we aimed to identify and characterize 29 isolates A. flavus. Morphological differentiation of A. flavus isolates belonging to maize grains may be few but it is difficult to define, due to interspecific significant similarities. Phenotypic characterization (morphological characteristics) is similar and subjective and dubious results limit accurate identification. In general, morphological characteristics are also commonly used to classify Aspergillus as this approach is important to categorize the isolates by similar groups, which allows used further methods to confirm identification. Morphological and biochemical characterization are usually complemented with a mycotoxigenic pattern of AFs to get more accurate identification (Rodrigues et al., 2009; Norlia et al., 2018).

Many scholars have attempted to establish a link between the capacity to generate sclerotia and the development of AF, but published evidence is paradoxical. There is a strong association between the presence of small sclerotia and high-level production of AF in different studies (Novas and Cabral, 2002; Pildain et al., 2004) while other researchers reported no relationship between sclerotial production or size and AF production [18,40] or an even reverse linkage, with large type isolates being the most aflatoxigenic (Abbas et al., 2005). Results can be contradictory because authors have not been establishing a standardized method for fungal growth conditions and media for this purpose (Rodrigues et al., 2009).
CAM fluorescence did not allow unequivocal discrimination of aflatoxigenic from non-aflatoxigenic *A. flavus* isolates, leading to false positives or false negatives in some of the tested isolates. Nonetheless but, CAM fluorescence a simple method for a fast screening for the isolates were potentially aflatoxigenic. Many authors have stated that CAM fluorescence is not reliably consistent with HPLC aflatoxin detection (Scherm et al., 2005; Giorni et al., 2007). Some authors have another opinion, detection of AF production by CAM fluorescence showed a good correlation with the HPLC results (Rodrigues et al., 2009; Norlia et al., 2018). Qualitative techniques like CAM fluorescence, ammonium vapor yellow pigment for the detection of AF production by *A. flavus* have been stated not to be reliable or accurate. Thus, besides traditional techniques, we need to use analytical or molecular techniques (Adetunji et al., 2019). From different biological sources, 166 *A. flavus* isolates were collected and tested by yellow pigmentation. The findings of analysis reported 152 isolates (92.5%) of these isolates produced AFs and 144 isolates (87%) were harmonious to HPLC analysis (Rao et al., 2020).

A. flavus isolates were collected from four agroclimatic zones of India and exposure cultures to ammonia vapour. 22% of isolates were highly aflatoxigenic isolates displayed plum red color, 12% moderately aflatoxigenic shown pink color, and 10% toxic isolates indicated to red color. The ammonia vapor test is simple, low-cost, accurate, and time-saving. The method that can be used to segregate or pre-screen contaminated samples from staple food or feedstocks (Shekhar et al., 2017). The ammonia vapor and yellow pigmentation have been color reaction is attributed only to intermediate anthraquinone metabolites such as norsolonic acid and other unique aflatoxin-related chemicals (Shier et al., 2005).

The culture methods are not as dependable as HPLC analysis, but they are easy, inexpensive. Cultural methods have been used for the detection of AFs production. They are more appropriate for the first screening with less time and fewer resources in a large population in potentially extreme *A. flavus* isolates. In some cases, it is not possible to depend on culture methods only, but we need a reliable method as HPLC analysis. Methods for the detection of aflatoxin production. They are most suitable for the first screening.

### Table 6

| No. | Isolate code | Aflatoxin B1 (HPLC; ng/mg mycelia dry weight) | Type<sup>c</sup> | Gene expression<sup>a</sup> |
|-----|--------------|---------------------------------------------|------------------|-----------------------------|
|    |              |                                             |                  | aflD | aflM | aflO | aflP | aflQ | aflR |
| 1   | AsF-mR11     | ND<sup>d</sup>                              | –                | –   | –   | –   | –    | –    | –    |
| 2   | AsF-mR12     | ND                                          | –                | –   | +   | +   | –    | +    | –    |
| 3   | AsF-mR13     | ND                                          | –                | –   | –   | –   | –    | –    | –    |
| 4   | AsF-mR14     | 53.8                                       | +                | –   | –   | +   | +    | –    | –    |
| 5   | AsF-mR15     | 76.1<sup>b</sup>                            | +                | –   | –   | +   | +    | +    | –    |
| 6   | AsF-mR16     | ND                                          | –                | –   | –   | –   | –    | –    | –    |
| 7   | AsF-mR17     | 34.5                                       | +                | –   | –   | +   | –    | +    | –    |
| 8   | AsF-mR18     | ND                                          | –                | –   | +   | +   | –    | –    | –    |
| 9   | AsF-mR19     | ND                                          | –                | –   | –   | +   | –    | –    | –    |
| 10  | AsF-mR20     | 57.4                                       | +                | –   | –   | +   | +    | +    | –    |
| 11  | AsF-mR21     | ND                                          | –                | –   | –   | –   | –    | –    | –    |
| 12  | AsF-mR22     | ND                                          | –                | –   | –   | –   | –    | –    | –    |
| 13  | AsF-mR23     | 23.2                                       | +                | –   | –   | +   | +    | +    | –    |
| 14  | AsF-mR24     | ND                                          | –                | –   | –   | –   | –    | –    | –    |
| 15  | AsF-mR25     | 27.7                                       | +                | –   | –   | +   | +    | +    | –    |
| 16  | AsF-mR26     | ND                                          | –                | –   | –   | –   | –    | –    | –    |
| 17  | AsF-mR27     | ND                                          | –                | –   | –   | –   | –    | –    | –    |
| 18  | AsF-mR28     | ND                                          | –                | –   | –   | +   | –    | +    | –    |
| 19  | AsF-mR29     | ND                                          | –                | –   | –   | –   | –    | –    | –    |
| 20  | AsF-mR30     | ND                                          | –                | –   | –   | –   | –    | –    | –    |
| 21  | AsF-mR31     | ND                                          | –                | –   | –   | –   | –    | –    | –    |
| 22  | AsF-mR32     | 94.7<sup>b</sup>                            | +                | –   | –   | +   | +    | +    | –    |
| 23  | AsF-mR33     | 50.9                                       | +                | –   | –   | +   | +    | +    | –    |
| 24  | AsF-mR34     | 36.4                                       | +                | –   | –   | +   | +    | +    | –    |
| 25  | AsF-mR35     | ND                                          | –                | –   | –   | –   | –    | –    | –    |
| 26  | AsF-mR36     | 97.2<sup>b</sup>                            | +                | –   | –   | +   | +    | +    | –    |
| 27  | AsF-mR37     | 63.8<sup>b</sup>                            | +                | –   | –   | +   | +    | +    | –    |
| 28  | AsF-mR38     | ND                                          | –                | –   | –   | –   | –    | –    | –    |
| 29  | AsF-mR39     | ND                                          | –                | –   | –   | –   | –    | –    | –    |

<sup>a</sup>A. flavus isolates type II were producing AFB1 in amounts ≤ 60.
<sup>b</sup>Fungal culture were grown in YES medium for four days at 28 °C.
<sup>c</sup>A. flavus isolates type I classified according to their ability for producing AFB1 in amounts ≥ 60 ng/ mg mycelia dry weight.
<sup>d</sup>ND = Not detected.

**Fig. 3.** RT-PCR amplification of 9 RNA of aflatoxigenic *A. flavus* isolates using specific primers of six genes of AF biosynthesis, including *aflD*, *aflM*, *aflO*, *aflP*, *aflQ* and *aflR*. C: Positive control, lane 1: AsFmR14, lane 2: AsFmR15, lane 3: AsFmR17, lane 4: AsFmR20, lane 5: AsFmR23, lane 6: AsFmR25, lane 7: AsFmR27, lane 8: AsFmR23 and lane 9: AsFmR36.
of a large community of potentially serious Aspergillus isolates in less time and limited resources (Rao et al., 2020). The HPLC system used for detection and measurement of AFs is specific, highly sensitive, and accurate in A. flavus isolates and well known in many agricultural crops, such as maize (Sserumaga et al., 2020), wheat (Al-Wadai et al., 2013), peanut, (Norlia et al., 2018) and spices (El-Dawy et al., 2019).

Assessment of four AF-genes PCR, aflD, aflO, aflP, and aflQ, were did not correspond with the aflatoxigenicity profile of A. flavus isolates (Abdel-Hadi et al., 2011; Jamali et al., 2013; Mahmoud, 2015). Multiplex RT-PCR molecular results for 11 genes (structural and regulation) in 13 A. flavus isolates concluded that the best correlation for aflatoxigenic character was for three expression genes. The expression profile of the three aflD, aflO, and aflP genes was consistently correlated with the capacity to produce AFs (Scherm et al., 2005). 31 A. flavus isolates were analyzed by the expression of two AF-genes, aflD, and aflQ. AFLQ expression demonstrated a significant correlation between the expression gene and the capacity to produce AFs, but aflD was a failure in this assignment (Rodrigues et al., 2009). The expression of four AF-genes (aflD, aflO, aflP, and aflQ) was estimated in twenty-four A. flavus isolates. Transcriptional profile of aflD and aflP genes provided these genes has been not associated with AFB1 production but, the transcriptase patterns of aflO and aflQ have been set to be perfectly correlated with the values of AFB1 produced (Jamali et al., 2013). Four AF-genes aflD, aflM, aflP, and aflQ were evaluated employing RT-PCR in 22 A. flavus isolates from peanut kernels. The transcriptic analysis of aflD and aflQ displayed a helpful marker for discrimination between toxigenic and atoxigenic A. flavus isolates (Mahmoud, 2015). Finally, there is currently no consensus for transcription data where a single gene should be used or more than one gene to differentiate entirely between producers of AF and non-AF, however many researchers have been needed to transcription analysis for more than one gene to get fully discriminate (Abdel-Hadi et al., 2011; Jamali et al., 2013; Mahmoud, 2015).

The expression of the aflR gene was analyzed by qRT-PCR and it appeared that the expression of the aflR gene was a gradual increase in A. flavus from maize and without non-specific expression from rice grain. The findings of qRT-PCR indicate an effective molecular tool for detecting and dominating A. flavus distribution in grain stores but the expression gene is highly related to the crop (Imran et al., 2014). SYBR Green qPCR of three AF-genes (aflM, aflR, and aflD) utilized for Early detection of maize contamination by AB1. qPCR protocol founded on these genes could be developed significantly related to the detection and determination of AB1 content in food or feed samples. qPCR is an advanced, accurate, and sensitive, a method for discovering expression gene in A. flavus (Anong et al., 2016). Transcriptional analysis was completed by qRT-PCR by utilizing specific AF-genes aflR, aflD, and aflP to control the production of AB1. aflatoxigenic A. flavus isolates exhibited a higher expression gene of aflR and aflD genes (p < 0.05). Our findings proposed that the expression of aflR and aflD is related to AB1 production in A. flavus and that overexpression of aflR could affect the toxigenic and transcriptional pattern (Baquião et al., 2016).

5. Conclusions

To classify and recognize aflatoxigenic and non-aflatoxigenic A. flavus isolates, a polyphasic approach involving morphological, biochemical, and molecular characterization was applied to 29 A. flavus isolates collected from stored maize in Riyadh City, Saudi...
Arabia. In two classes, the aflatoxigenicity profile of A. flavus isolates by HPLC was involved, first with 38% being aflatoxigenic isolates and second with 62% being non-aflatoxigenic isolates. The expression pattern of aflO and aflQ was a beneficial marker for discriminating aflatoxigenic from non-aflatoxigenic A. flavus isolates. Also though the pathway of AF biosynthesis is complex, but a strong link has been observed between the expression of the gene and production of aflatoxin, qRT-PCR of AF-genes present an important role in the field.

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Declaration of Competing Interest
The authors declared that there is no conflict of interest.

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