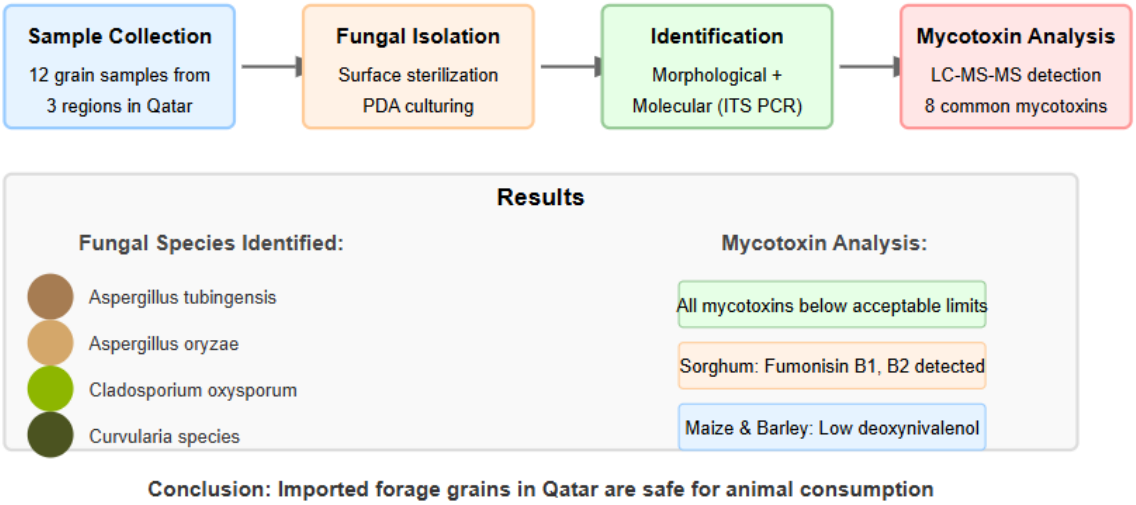
Molecular and Morphological Identification of Mycotoxigenic Fungi in Imported Forage Cereals and Safety Assessment in Qatar

Mohammed Mazen[1,2*], Hamad Al-Shamari[1], Sowaid A. Al-Maliki[1], Talaat Abdelfattah Youssef [3], Sheeja Thomas[1], Amjad Mohmoud[3]

[1]Department of Agricultural Research, Ministry of Municipality, Doha 2727, Qatar.
[2]Agricultural Research Center, Al Giza, Giza Governorate 3725004, Egypt.
[3]Environmental Science Centre, Qatar University, Doha 2713, Qatar.

Graphical Abstract



Abstract

This study aimed to identify and quantify fungi and mycotoxins in imported forage grains (sorghum, maize, and barley) stored in Qatar to assess potential health risks. The finding of this study reports the type and levels of fungi and mycotoxins associated with these grains imported to Qatar from different countries. A total of twelve-grain samples collected from different regions in Qatar were analyzed. All the samples were found to contain one or more fungal genera. The isolated species were identified morphologically as *Aspergillus* spp, *Cladosporium* sp, *Curuvalria* sp, *Rhizopus* sp, and one unidentified genus. The fungal isolates were then identified at the species level through molecular techniques. DNA sequencing followed by BLAST (Basic Local Alignment Search Tool) analysis identified the species as *Aspergillus tubingensis*, *Aspergillus oryzae*, *Cladosporium oxysporum*, *Curvularia chonburiensis*, and *Curvularia lunat.* By using molecular techniques, the process of distinguishing between similar species of fungi was significantly enhanced over traditional methods. Based on the prevalence of potentially mycotoxigenic fungi, representative samples from sorghum (sample-6), maize (sample-4), and barley (sample-7) were analyzed for eight common mycotoxins, including aflatoxins, ochratoxin A, and fumonisins. Concentrations of the tested mycotoxins in the samples were all below the acceptable limit. Most mycotoxins were below detection limits, suggesting that the current import and storage practices for these grains in Qatar effectively prevent mycotoxin accumulation to harmful levels.

Keywords: Mycotoxigenic fungi; Forage cereals; Molecular identification; Food safety; Storage conditions.

1. Introduction

Mycotoxins are toxic secondary metabolites produced by various fungi that contaminate food and feed commodities, particularly cereals. These compounds pose significant health risks to humans and animals, causing adverse effects ranging from acute poisoning to long-term consequences such as carcinogenic, mutagenic, teratogenic, and immunosuppressive effects (Kumar et al., 2021).

The State of Qatar imports large quantities of cereal grains (sorghum, barley, and maize) to maintain the strategic stock of animal feed and human consumption, which is critical for ensuring national food security. As these grains are stored for several months, ensuring their quality and safety from fungal contamination and mycotoxin production is essential. Various types of fungi can attack grains both in the field and during storage, causing them to grow and produce mycotoxins under suboptimal conditions (Mahato et al., 2019 & Krnjaja et al.,2017).

Mycotoxins-producing models flourish in hot environments and sub-tropical climates, creating favorable conditions for fungi growth (Misihairabgwi , J. M et al., 2017). Among the world's driest regions, the Arabian Gulf has high salinity levels and little precipitation (Roda F. Al-Thani & Bassam T. Yasseen. 2021). Qatar experiences a subtropical dry climate with very high summer temperatures and periods of prolonged humidity. These conditions are conducive to mold growth and the subsequent contamination of stored grains with mycotoxins (Rodrigues & Chin, 2012. & Okun et al., 2015). Fungal contamination can occur during pre-harvesting, handling, transporting, or storing, fungal growth and molds are most likely to contaminate forage grain. Such contamination can adversely affect humans and livestock, potentially leading to severe or chronic health problems (Moss, 1996; &Kehinde et al., 2014).

Mycotoxins are secondary metabolites synthesized by fungi that are toxic to humans and animals, even at low concentrations. Toxin production is inconsistent and depends primarily on environmental conditions, with grain mold typically caused by one or more fungal species, depending on locality, entry point, and storage conditions. Over 200 mycotoxin types have been identified, including aflatoxins, ochratoxins, citrinin, patulin, and fusarium toxins, each with varying levels of toxicity. In forage grains, this toxin can cause harmful biochemical changes, including reduced carbohydrates and oil content and increased free fatty acids affecting nutritional quality and safety (Reddy et al., 2010; Alkahtani et al., 2011; Speijers & Speijers, 2004).

Several fungi genera are prominent in stored forage grains, including *Aspergillus, Penicillium, Fusarium, and Alternaria*, among the most prevalent post-harvest fungal contaminants. These fungi can be categorized as field fungi and storage fungi, as they can grow in both environments when conditions are favorable (Belkacem-Hanfiet al.,2013). Mycotoxin production in forage grains depends on both the environmental conditions and the toxigenic potential of the fungi present, with mycotoxin release occurring when the storage conditions are not properly monitored. Consequently, both livestock and humans can be adversely affected by fungi and mycotoxins in contaminated grains or silage (Penagos-Tabares et al.,2022). Proper handling practices, early detection methods, and optimal storage conditions are essential to prevent mycotoxin contamination and ensure food security. Accurate identification of mycotoxigenic species and their toxins is crucial before assessing their potential impact on livestock (Sardiñas et

al., 2011). Therefore, Qatar requires established methods for regularly assessing and screening mold presence on storage grains.

The analytical method should be fast, less labor-intensive, and affordable while effectively identifying mycotoxigenic fungal strains. Molecular identification methods such as Polymerase Chain Reaction (PCR) based assay permit very sensitive, rapid, and specific detection of mycotoxigenic fungi from pure cultures as well as directly in feed crops (Gil-Serna et al., 2013; Jurado et al., 2006; Sampietro et al., 2010). The selection of an appropriate protocol for sampling mycotoxins is very crucial because they occur at low concentrations. The EU Regulation No 691/2013 is one of the most well-known standard methods for sampling animal feed crops for mycotoxins. This standard method collects a representative sample, homogenized, and ground to powder. Then, a sub-sample of known weight is extracted using a suitable solvent. Most mycotoxins are polar and organic; therefore, they are soluble in polar organic solvents, including acetonitrile, methanol, acetone, chloroform, and dichloromethane. Extraction can be done using simple solvent extraction, microwave-assisted extraction, ultrasonic extraction, and other more advanced analytical techniques (Pereira, Cunha et al. 2019). Extracted metabolites can then be analyzed chemically (chromatography: TLC, GC, GC-MS, LC-MS) or biologically (e.g., immunoassay-based methods).

Therefore, this study aims to (i) inspect the fungal species contaminating forage grains used in animal husbandry in Qatar, (ii) identify the most common types of fungi causing grain contamination, and (iii) use an integrated approach that combines traditional culturing method and DNA-based technique to identify mycotoxigenic fungi, along with LC-MS-MS detection of mycotoxins.

2. Materials and Methods

2.1 Collection of Forage Cereal Samples

Twelve samples of one kilogram of each forage grain (sorghum, barley, and maize) were collected from the storage farms in three different regions (North, South, and Central regions) within the State of Qatar, as listed in **Table 1**. Samples with proper labeling were transported to the Plant Pathology and Protection Laboratory at the Agriculture Research Laboratory, Ministry of Municipality. All samples were stored at 4 °C until mycological analysis was conducted.

2.2 Analysis of the Mycoflora - Fungal Isolation and Identification

2.2.1 Enumeration of Fungal Colonies

The standard test method recommended by the International Commission on Food Mycology (ICFM) was employed to isolate and purify fungal species (Hocking et al., 2006). This procedure involves the following steps:

(i) *Surface Disinfection*: This process removes surface contaminants from dust and other sources while allowing recovery of existing fungi. One hundred seeds from each forage grain sample were randomly selected and immersed in 0.4% sodium hypochlorite solution for two minutes with continuous stirring. Seeds were then rinsed with sterile distilled water for one minute and dried on sterile filter paper before direct plating. This disinfection procedure was repeated three times.

(ii) Plating: Seven surface-sterilized seeds of sorghum and five surfaced-sterilized seeds of each maize and barley were placed on separate 90 mm diameter Petri dishes containing Potato Dextrose Agar (PDA). All tests were performed in duplicate for each sample.

(iii) *Incubation*: Petri plates were sealed with para-film® and incubated upright for 5-10 days at 28.0 ± 2.0 °C. Distinct colonies representing different fungal species were subsequently sub-cultured.

(iv) *Purification of fungal species*: Spores or mycelium fragments from distinct colonies were inoculated onto fresh PDA medium. This process was repeated until pure cultures were obtained. The pure cultures were then maintained on a PDA slant, coded for identification, and stored at 4°C.

(v) *Morphological identification of purified fungi:* Pure fungal cultures were identified based on colony morphology and microscopic examination (Barnett & Hunter, 1972; Leslie & Summerell, 2006).

Table 1. Samples were collected from different farms covering Qatar's northern, southern, and central regions.

Sample No.	Forage grain	Location	Area
1	Sorghum	North	Al Shaflahiyah Farm No. 1132
2	Sorghum	North	Al Shaflahiyah Farm No. 194
3	Sorghum	South	Al Shahaniya, Farm No. 972
4	Maize	North	Al-Ghosn Farm No. 174
5	Maize	Central	Umm Salal Farm No. 204
6	Sorghum	South	Al Shahaniya Farm No. 363
7	Barley	North	Al Shaflahiyah Farm No.189
8	Barley	North	Al-Ghosn Farm No. 181
9	Barley	South	Al Shahaniya Farm No. 538
10	Barley	South	Al Shahaniya Farm No.237
11	Barley	Central	Umm Salal Farm No. 1253
12	Barley	Central	Umm Salal Farm No. 1184

The percentage of infected forage grains with a specific fungal genus was calculated using the following formula described by Marasas *et al*. (1988):

a. Infected seeds per sample (%) = (No. of infected seeds by a genus/ Total number of seeds) × 100.

b. Relative frequency of contaminated samples (Fq) (%) = (No. of samples contaminated with a specific genus/Total number of samples) × 100.

c. Relative density Rd (%) = (No. of isolates of a specific genus contaminating a sample/Total number of isolates) × 100.

2.3 Molecular Identification of the Isolated Fungi

2.3.1 Extraction of Fungal Genomic DNA

Pure mycelial fungal isolated cultures grown on PDA plates were collected from the Plant Pathology and Protection Laboratory at the Agricultural Research Department, Ministry of Municipality. Total fungal genomic DNA was isolated from mycelial growth overnight in a PDA medium. The DNA was extracted using the DNeasy Plant Kit (Qiagen) following the DNeasy® Plant Handbook instructions. Fungal mycelial was ground in liquid nitrogen using mortar and pestle to obtain fine powder. 100 mg of this ground fungal myceliam was used for DNA extraction.

2.3.2 PCR Amplification

Fungal total genomic DNA was subjected to 18S rDNA PCR partial amplification. The ribosomal DNA (rDNA) ITS region of the extracted DNA was amplified with ITS1 (5'-TCCGTAGGT GAACCTGCGG-3') and ITS4 (5'-CCTCCGCTTATTGATATGC -3') universal primers. The total volume of the PCR solution was 25µl, 2 µl of DNA (25 ng/ µl) as a template DNA, 10 µM dNTP, 5 µM of both ITS1 and ITS4 primer, 1.5 mM MgCl, 1X buffer, and finally 1 unit of *Taq polymerase* was added. PCR amplification was carried out with the thermal cycler (Applied Biosystems) at 94°C for 4 min for (initial denaturation) followed by 35 cycles as follows: 1 min at 94°C (denaturation), 2 min at 55.5°C (annealing), 72°C for 2 min (extension), and the final extension at 72°C for 10 min.

2.3.3. Agarose gel electrophoresis

PCR-amplified products were checked by electrophoresis on a 2% (w/v) agarose gel stained with sybr safe stain. A volume of 5 µl of PCR product and 1 µl of loading dye loaded for each sample. An appropriate ladder DNA marker was run in parallel with the PCR-amplified product (White et al., 1990). The quantity of PCR products was determined using NanoDrop.

2.3.4. DNA Purification

Following PCR amplification, PCR products were purified using the PureLink Quick Gel Extraction Kit (cat. no. K210012). Desired fragments of intact DNA from TBE agarose gels (derived from gel electrophoresis of amplified samples) were isolated by a series of filtration steps. The ethanol wash provided in the purification kit allowed the impurities and salt to be washed out while maintaining the desired DNA in the spin column.

2.3.5. DNA sequencing

PCR products of ITS regions of rDNA were sent to the genomics core laboratory of Weill Cornell Medicine – Qatar for sequencing. A 16- capillary 3100 Genetic Analyzer (Applied Biosystems) was used. The ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit Version 3 (Applied Biosystems) was used with protocols supplied by the manufacturer. PCR-amplified products from the fungal isolates were sequenced forward and reverse using ITSl primer and ITS4 primer, respectively. Sequences were visualized and edited using Chromas Version 1.45; Technelysium Pty. Ltd.

2.3.6 Gene Bank Data

To obtain homology with sequences of identified fungal species, a sequence search was performed using the BLAST standard nucleotide-nucleotide basic local alignment search tool [National Center for Biotechnology Information (NCBI), Library of Medicine, Bethesda, MD, USA (http://www.ncbi.nlm.nih.gov/BLAST/)].

2.4 Chemical Analysis of Mycotoxins

Representative samples of about three kilograms of each of Sorghum (Sample 6), Maize (Sample 4), and Barley (Sample 7) were collected based on their higher fungal contamination levels as determined by the initial screening. The samples were homogenized by mixing before getting representative subsamples of approximately 200 g each. The subsamples were ground to powder using a grinding machine. The powdered samples were stored in plastic bags in the dark at -20 °C until analysis. One gram of the powder of each sample was accurately weighed and extracted with acetonitrile/methanol(1:1) mixture and mixed with ammonium format. The extracts were evaporated to dryness under a gentle stream of nitrogen. The residues were reconstituted with methanol/water (1:1) to 1 mL, filtered through a 0.22 µm nylon micro syringe filter, and finally analyzed by LC-MS-MS for eight common mycotoxins including aflatoxins (B1, B2, G1, G2), *ochratoxin A*, *fumonisin* B1, *deoxynivalenol*, and *zearalenone*. The LC-MS-MS analysis was performed using a Thermo Scientific Vanquish UHPLC system coupled to a TSQ Altis triple quadrupole mass spectrometer with a Kinetex C18 column (100 × 2.1 mm, 2.6 µm particle size). The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), and the flow rate was 0.25 mL/min. The MS detection was performed in positive and negative electrospray ionization (ESI+/ESI-) modes with polarity switching with the following parameters: spray voltage, 3500 V (positive) and 2500 V (negative); sheath gas, 45 arbitrary units; auxiliary gas, 15 arbitrary units; ion transfer tube temperature, 325°C; vaporizer temperature, 350°C; and collision gas pressure, 1.5 mTorr. Multiple reaction monitoring (MRM) mode was used for quantification with two transitions monitored for each mycotoxin, with the most abundant product ion used for quantification and the second most abundant for confirmation.

2.5. Quality control procedures

All media, reagents, and materials used for fungal isolation and molecular analysis were sterilized by autoclaving at 121°C for 15 minutes. All fungal isolation experiments included negative controls (non-inoculated media) to check for environmental contamination. For PCR analysis, negative controls (no template DNA) and positive controls (DNA from reference fungal strains) were included in each PCR run to ensure the reliability of the results.

2.6.Statistical analysis

Data on fungal contamination rates and mycotoxin concentrations were analyzed using descriptive statistics (mean, standard deviation, and percentage). Correlation analysis was performed to examine relationships between fungal contamination levels and mycotoxin concentrations. All statistical analyses were performed using SPSS version 26.0 (IBM Corp., Armonk, NY, USA), with $p < 0.05$ considered statistically significant.

3. Results

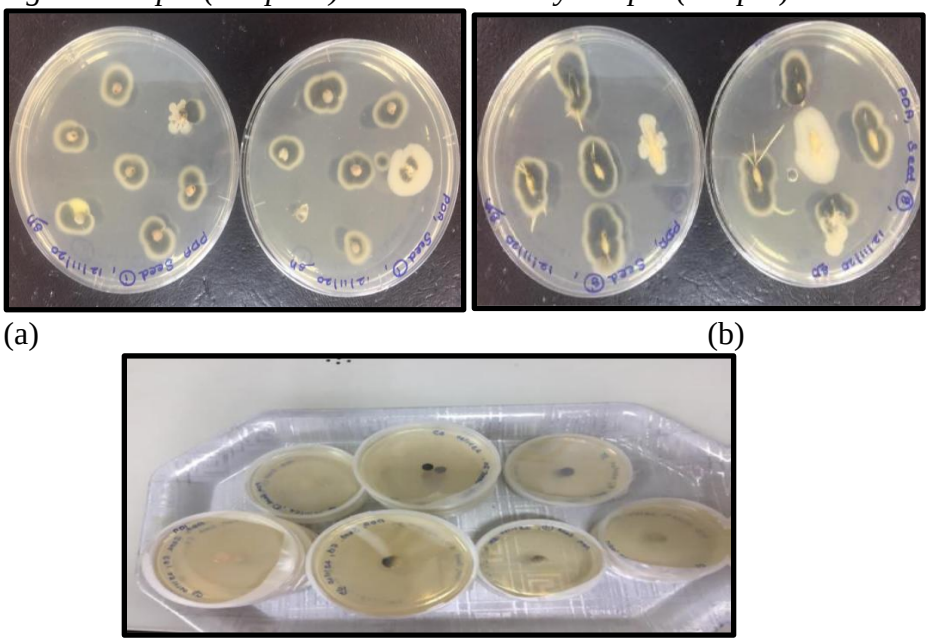
3.1 Enumeration, Seed Surface Disinfection, Plating, and Purification of Fungal Species

The surface of each sample was sterilized, dried on sterile filter paper, and placed on a Potato Dextrose Agar (PDA) plate. The plates were incubated for 5-10 days at 28.0 ±2.0 °C. After the incubation period, diverse fungal colonies developed on each sample plate (**Figure 1**). Each of the fungal colonies were aseptically transferred to fresh PDA for morphological and microscopical examination.

3.2 Fungal Isolation and Microscopic Examination:

The investigated samples contained Aspergillus spp-1&2, Cladosporium sp., Curvularia sp-1&2, Rhizopus sp., and one unidentified genus. Aspergillus spp. had the highest frequency of occurrence, followed by Cladosporium sp. and Curvularia sp. (**Table 2**).

In sample 6 (Sorghum), four different fungal isolates were identified. The predominant fungi were *Aspergillus* (two distinct isolates), *Curvularia sp1*., and an unidentified fungal isolated with light brown mycelium. *Cladosporium sp. was detected in several samples, including sorghum (samples 1, 2, and 3), maize (samples 4 and 5),* and barley (samples 7 and 8). *Aspergillus sp.* (isolate 2) *and Rhizopus sp. were found in all barley samples, while Aspergillus sp (isolate 1). was detected only in one sorghum sample (sample 6) and in the Barley sample (sample*).



(a) (b)

(c)

Figure 1. Purification of fungal species isolated from different seed samples; (a) Fungal species isolated from sorghum seeds; (b) Fungal species isolated from barley seeds; and (c) Purification of fungal species on Potato Dextrose Agar (PDA)

Table 2. Fungal isolation and morphological characteristics of isolation from collected seed samples

Sample	Different fungi were isolated and identified under the Microscope.
Sorghum Sample 1	*Cladosporium* sp. covered the entire Petri plate.
Sorghum Sample 2	*Cladosporium* sp., *Curvularia sp-1., Aspergillus ssp-1*
Sorghum Sample 3	*Cladosporium sp., Curvularia sp-1.*
Maize Sample 4	Cladosporium sp., Curvularia sp-2.
Maize Sample 5	*Cladosporium* spp., *Curvularia spp2.*,
Sorghum Sample 6	*Aspergillus spp-1, Aspergillus spp-2, Curvularia sp-1*. Unidentified fungi exhibiting light brown color mycelium were recorded.
Barley Sample 7	*Cladosporium sp., Aspergillus spp-2.*
Barley Sample 8	*Cladosporium sp., Aspergillus ssp-1.*
Barley Sample 9	*Aspergillus ssp-2., Rhizopus sp.* covered all over the plate
Barley Sample 10	*Aspergillus ssp-2., Rhizopus sp*. covered all over the plate
Barley Sample 11	*Aspergillus ssp-2., Rhizopus sp*. covered all over the plate
Barley Sample 12	*Aspergillus ssp-2., Rhizopus sp*. covered all over the plate

3.2.1 Internal Mycoflora of Sorghum, Barley, and Maize Samples from Qatar:

The fungal genera's relative density and infection frequency in each sample were determined and recorded. The data are presented in **Figure 2.**

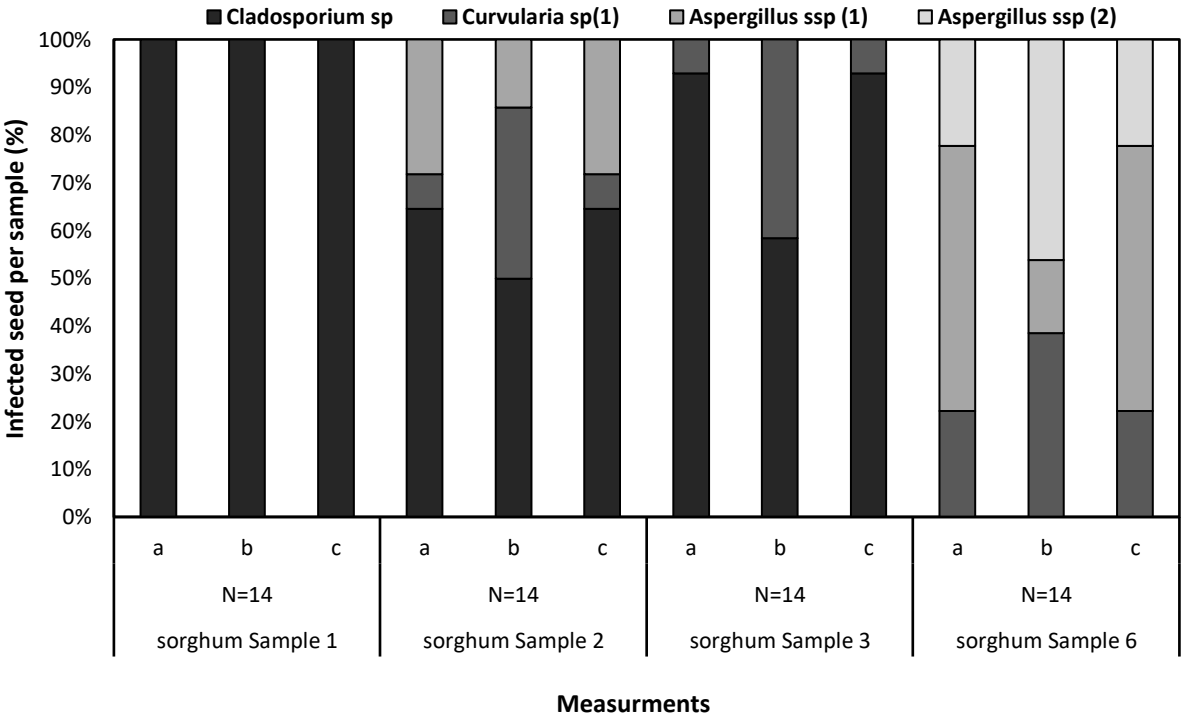


Figure 2. The internal microflora of sorghum seed samples was collected from different regions of Qatar; N = number of seeds per sample, a = infected seeds per sample (%) = (no. of infected seeds by a genus/ Total number of seeds) × 100, b = relative frequency of contaminated samples (Fq) (%) = (no. of samples contaminated with a specific genus/Total number of samples) × 100, **c** = relative density Rd (%) = (no. of isolates of a specific genus contaminating a sample/Total number of isolates) × 100.

Table 3 presents the fungal isolates associated with 12 samples of forage grain seeds collected from three different regions of Qatar. According to the results of our study, sorghum sample No. 6 was the most infected one among all, showing different species of fungi: *Aspergillus spp-1., Curuvlaria sp-1., Aspergillus spp-2. and other unidentified genera with the infection percentage value of 35.7 %, 28.57 %, 21.42 %,* and 14.33%, respectively. It was followed by sorghum sample No. 2, showing three different genera of fungi: *Cladosporium sp., Aspergillus spp-1., Curuvlaria sp-1.,* with an infection percentage of 64.3 %, 28.14 %, and 7.14 %, respectively. Sorghum sample No. 1 was infected with only one genera, *Cladosporium sp*. No other fungi were reported in the sample. In sorghum sample No. 3, Cladosporium sp. infected most seeds, 92.9 %, followed by *Curuvlaria sp-1.,* 7.14 %. (**Figure 3**)

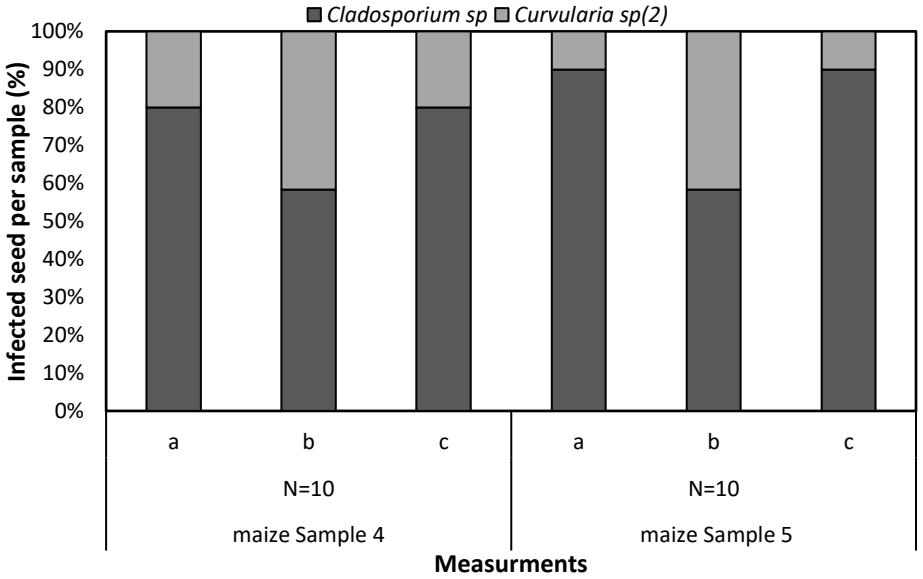


Figure 3. Internal mycoflora of maize samples (4 & 5).

Maize samples 4 and 5 showed the infection by two genera of fungi, Cladosporium sp. and *Curuvlaria sp*-2. The percentage of infection by *Cladosporium* was 80% and 90% in maize samples 3 and 4, respectively, followed by *Curuvlaria sp*-2., 10% and 20% (Figure 3).

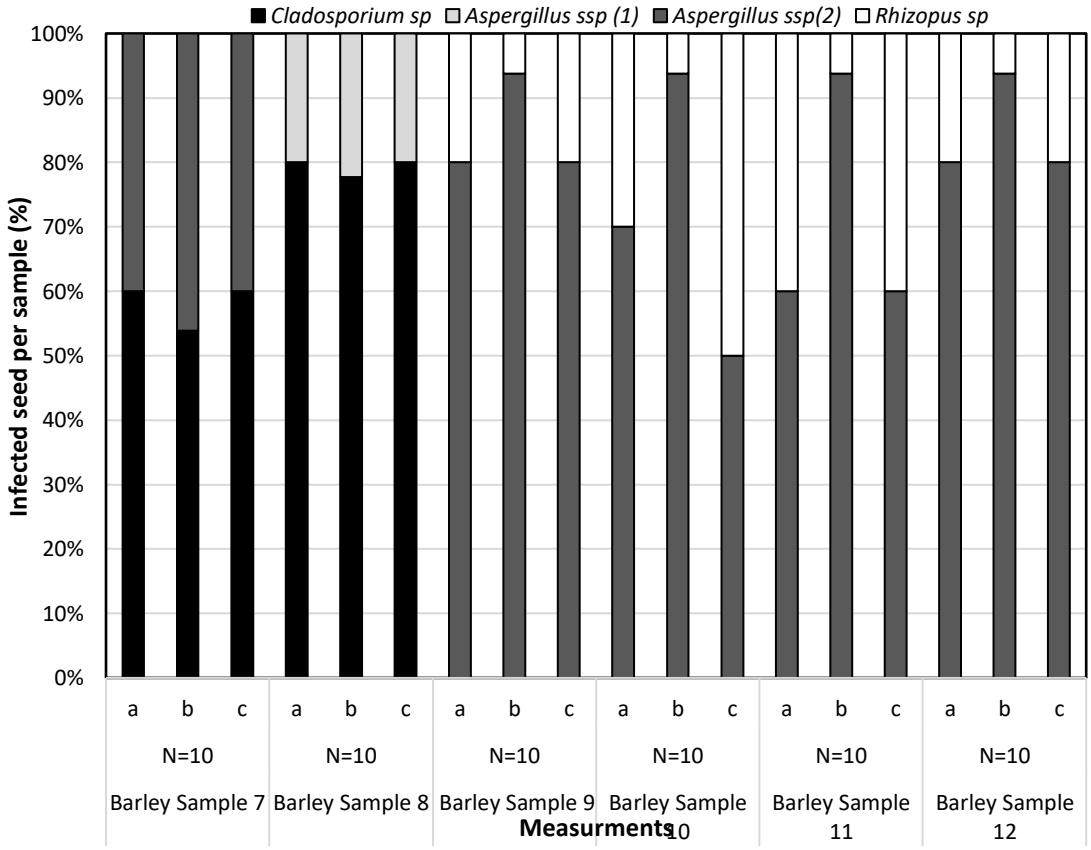


Figure 4. Internal mycoflora of barley sample (7, 8, 9, 10, 11 & 12)

The most observed fungi in barley samples were *Aspergillus ssp-2* and *Rhizopus sp*., The major infection in barley sample No 7 was caused by *Cladosporium sp.*, 60%, followed by *Aspergillus ssp-2.*, 40%. The percentage of *Cladosporium sp.*, and *Aspergillus ssp-2*. Contamination in barley sample No 8 was 80% and 20%, respectively. In barley sample 9, the recorded percentage of contamination by *Aspergillus ssp-2* and *Rhizopus sp*., was 80 and 20 respectively. *Aspergillus ssp-2*., and *Rhizopus sp.*, were recorded in barley samples 10, 11 & 12. (**Figure 4**).

3.2.2 Microscopic identification of fungal isolates isolated from sorghum, barley, and maize samples:

In each sample, purified fungal isolates were examined under a microscope and their morphology was recorded (**Figure 5**).

Fungal total genomic DNA was extracted from 5 pure fungal cultures, namely from one *Cladosporium sp*., two *Curvularia sp*., and two *Aspergillus spp*. Extracted DNA was used as template DNA in the ribosomal DNA (rDNA) ITS region PCR partial amplification. The sizes of amplified bands were around 500 bp (**Figure 6).**

PCR products of ITS regions of rDNA were sent to the genomics core laboratory of Weill Cornell Medicine – Qatar for sequencing. Sequences were visualized and edited using Chromas Version 1.45; Technelysium Pty. Ltd.

Sequence search was performed using the BLAST standard nucleotide-nucleotide basic local alignment search tool [National Center for Biotechnology Information (NCBI), Library of Medicine, Bethesda, MD, USA (http://www.ncbi.nlm.nih.gov/BLAST/)] to obtain the homology with sequences of identified fungal species.

Molecular identification of the pure fungal culture confirmed the microscopic identification done using light microscope. Moreover, using the ribosomal DNA (rDNA) ITS region sequencing could identify the genus and species. This was very helpful as only geniuses could be identified by using light microscope. For the first DNA sample, *Cladosporium oxysporum* was identified. However, for the two samples of *Curvularia* sp., two species were identified: *Curvularia chonburiensis* and *Curvularia lunata*. In the case of the two samples of two *Aspergillus ssp.* two species were identified: *Aspergillus tubingensis* and *Aspergillus oryzae* (**Table 3**).

Table 3. Molecular identification of the pure fungal cultures using ITS primers

Sample No.	Isolate	Identity	Accession No.	Similarity (%)
1.	Clad	*Cladosporium oxysporum*	NR_152267.1	99.21%
2.	Cur-1	*Curvularia chonburiensis*	NR_168176.1	99.02%
3.	Cur-2	*Curvularia lunata*	NR_138223.1	99.59%
4.	Asp-1	*Aspergillus tubingensis*	NR_131293.1	95.37%
5.	Asp-2	*Aspergillus oryzae*	NR_135395.1	98.95%

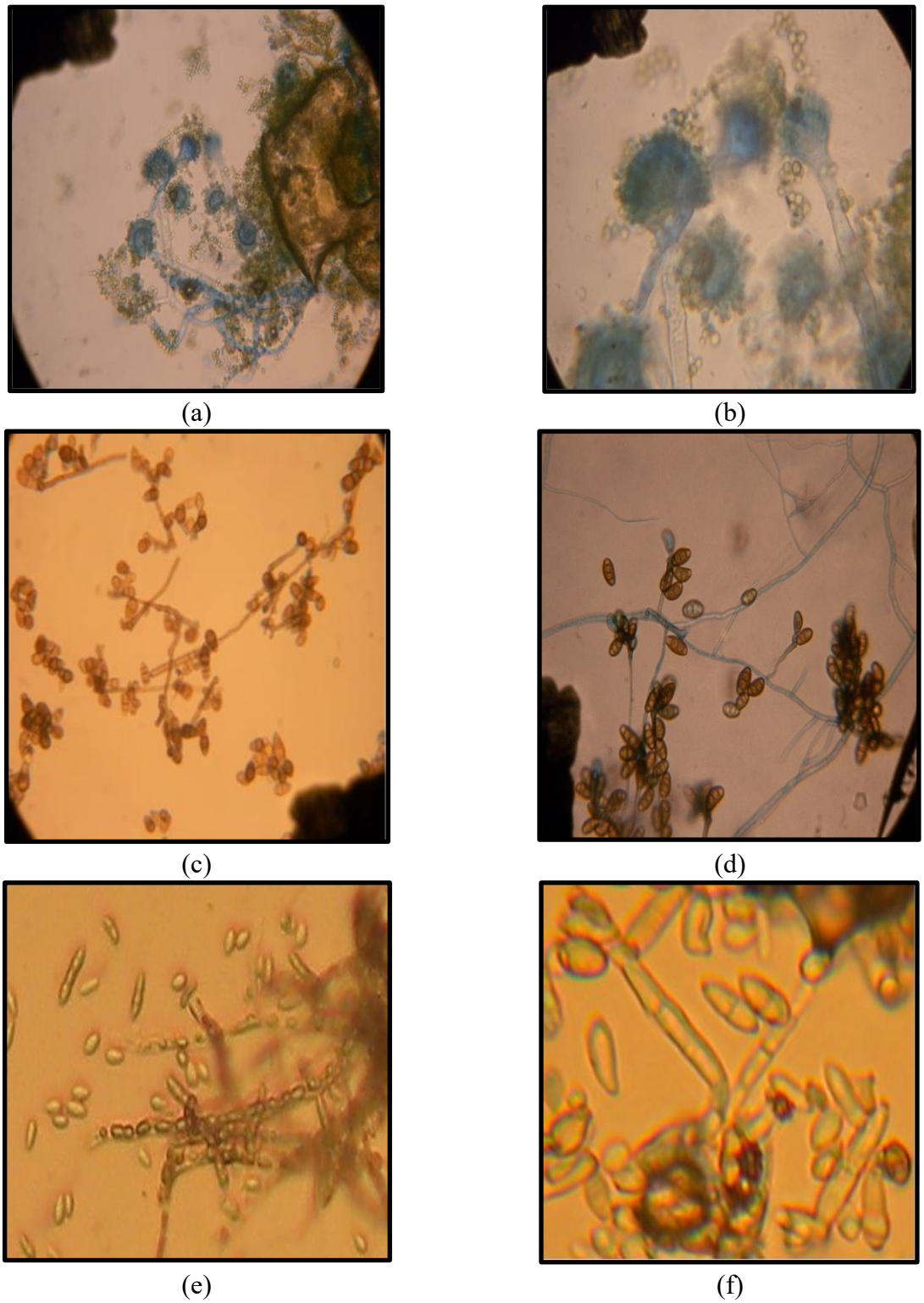


Figure 5. Microscopic identification of purified fungal isolates (5a. and 5.b *Aspergillus spp*; 5.c and 5.d *Curvularia sp*; 5. e and 5.f *Cladosporium sp.).*

3.3 Molecular identification of Fungal isolates

PCR amplification of the ribosomal DNA (rDNA) ITS region was performed on total genomic DNA extracted from five pure fungal cultures: one *Cladosporium sp.*, two *Curvularia* sp., and two *Aspergillus* spp. The amplification yielded distinct bands of approximately 500 bp size as shown in Figure 6. These results confirm successful amplification of the ITS regions from all five fungal isolates. The amplified PCR products were subsequently purified and sequenced for molecular identification of the isolated fungi at the species level. Sequence analysis through BLAST confirmed the microscopic identification and further distinguished the specific species within each genus, revealing *Cladosporium oxysporum*, *Curvularia chonburiensis*, *Curvularia lunata*, *Aspergillus tubingensis*, and *Aspergillus oryzae* as identified in **Table 3.**

3.4 Chemical analysis of Mycotoxins

Three of grain samples, namely sorghum (sample 6), maize (sample 4), and barley (sample 7), were analyzed for a range of mycotoxins (**Figure 7**) in the Central Food Laboratories Section – Ministry of Public Health, Qatar. The results are listed in **Table 4**.

Table 4. Concentrations of tested mycotoxins in the forage grain samples.

Parameter tested	Concentration [a]			LOD [a, b]	Acceptance limit [a]
	Sorghum (sample 6)	Maize (sample 4)	Barley (sample 7)		
Aflatoxin G2	<LOD	<LOD	<LOD	4	≤20.00
Aflatoxin G1	<LOD	<LOD	<LOD	4	≤20.00
Aflatoxin B2	<LOD	<LOD	<LOD	4	≤20.00
Aflatoxin B1	<LOD	<LOD	<LOD	4	≤20.00
HT-2 toxin	<LOD	<LOD	<LOD	40	n.a. [c]
Fumonisin B1	566.8	<LOD	<LOD	40	n.a. [c]
T-2 toxin	<LOD	<LOD	<LOD	40	n.a. [c]
Fumonisin B2	78	<LOD	<LOD	40	n.a. [c]
Ochratoxin A (OTA)	<LOD	<LOD	<LOD	2	≤5.00
Zearalenone	<LOD	<LOD	<LOD	40	≤75.00
Deoxynivalenol	<LOD	112.8	153.2	40	≤2000.00
Total Aflatoxin	<LOD	<LOD	<LOD	4	≤20.00
Total fumonisin (B1 + B2)	644.8	12.92	<LOD	n.a. [c]	≤2000.00
Total (HT-2 + T-2)	<LOD	<LOD	<LOD	n.a. [c]	≤50.00

[a] concentration in $\mu g.kg^{-1}$, [b] limit of detection, [c] not available.

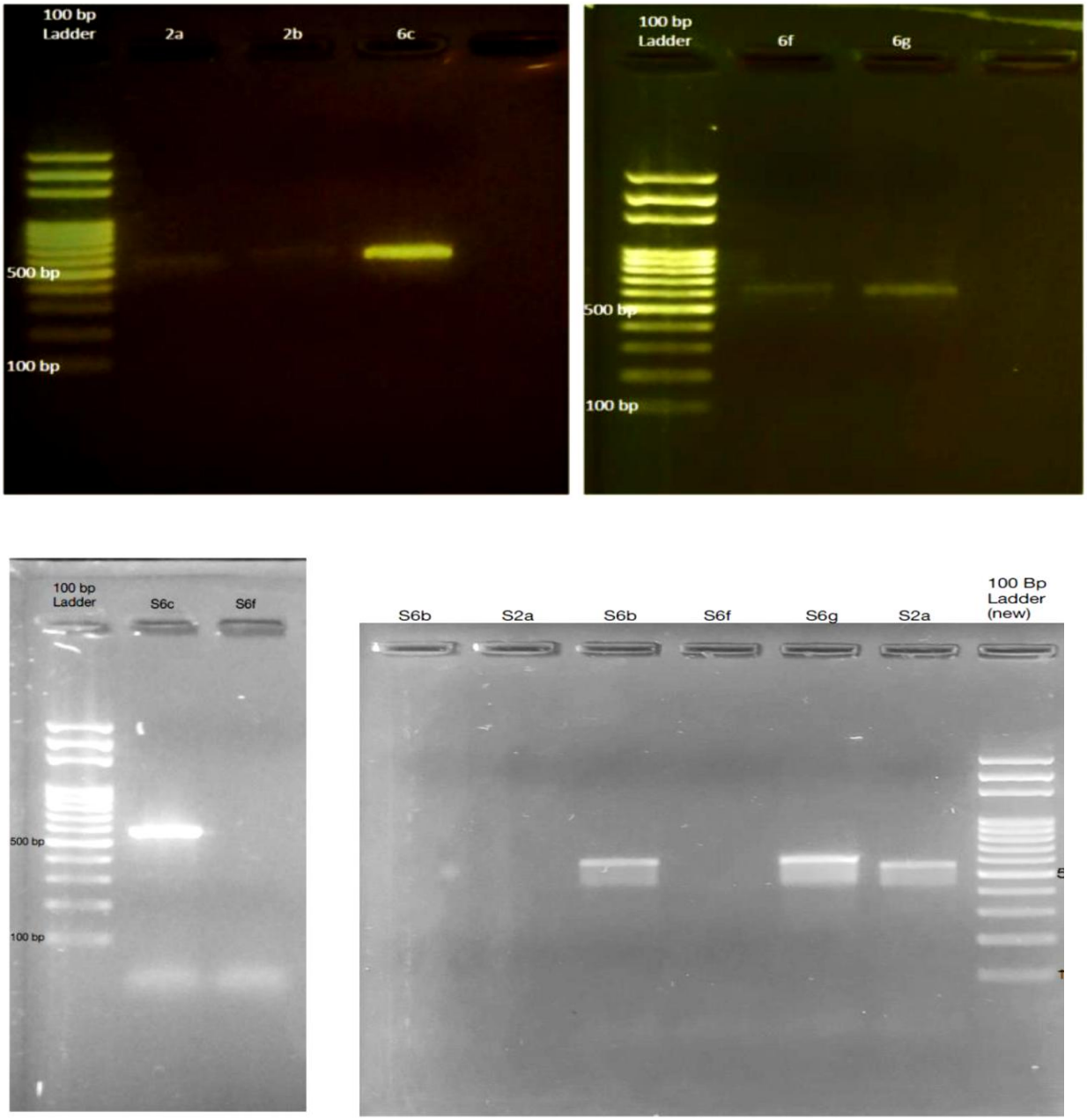


Figure 6. PCR partial amplification of the ribosomal DNA (rDNA) ITS region from pure Fungal culture of different samples.

Concentrations of tested mycotoxins in the samples were all below the acceptable limit, in fact most of the samples contained undetectable concentrations of the mycotoxins. Sorghum was found to contain fumonisin B1 at a concentration of 566.8 µg/kg, fumonisin B2 at a concentration of 78 µg/kg, and total fumonisin (B1 + B2) at a concentration of 644.8 µg/kg. Maize was found to contain deoxynivalenol at a concentration of 112.8 µg/kg and Total fumonisin (B1 + B2) at a concentration of 12.92 µg/kg. Barley, on the other hand, was found to have only deoxynivalenol at

a concentration of 153.2 µg/kg. Detected concentrations of mycotoxins in the analyzed samples were low to cause any harm to livestock.

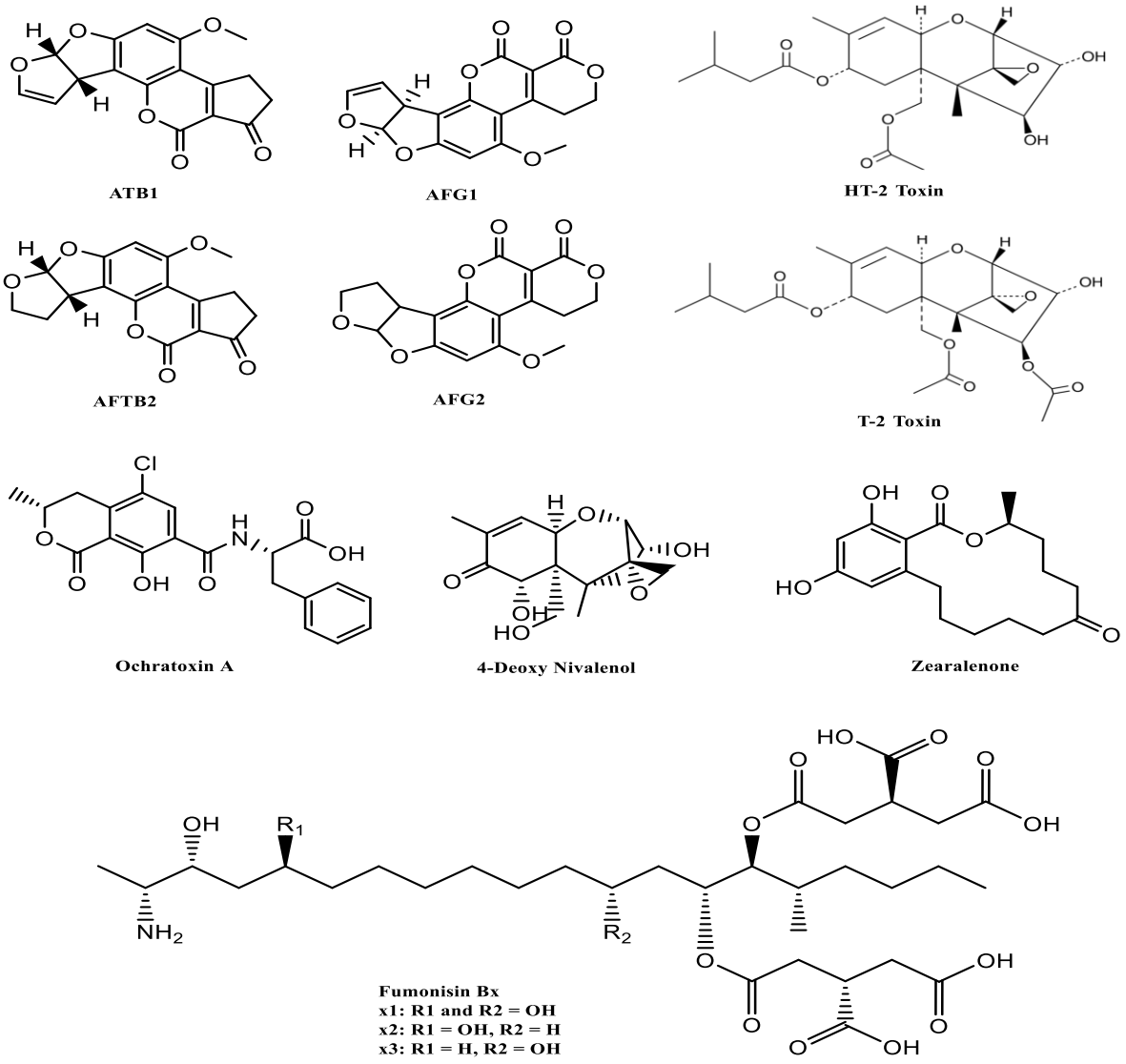


Figure 7. Structure of analyzed mycotoxins.

4. Discussion

Grain and other staple foods and feedstuffs containing mycotoxins can pose serious consequences for animal and human health. Many countries have established regulations defining maximum permissible levels of mycotoxins in food and feed products. Commodities exceeding these specified limits are prohibited from importation into most developed countries (Romina Alina Marc, 2022 and Chinaza Godswill Awuchi, 2021). Qatar imports large quantity of grains to meet the country's requirement, which raises significant concern regarding the safety of these grains

from fungal and mold infection that could threatens grain quality and compromise the country's food safety and security standards.

In this study, we analyzed 12 samples of sorghum, maize and barley collected from different regions of Qatar and identified several fungal genera. Mycological analysis revealed that all samples were contaminated to various degrees with storage fungi, while mycotoxins levels were below acceptable level. Molecular identification confirmed the presence of five species belonging to three different fungal genera: *Aspergillus tubingensis, Aspergillus oryzae, Cladosporium oxysporum, Curvularia chonburiensis,* and *Curvularia lunat.* Some unidentified fungi were also detected during the investigation. Similar mycoflora associated with sorghum have been reported by (Ackerman et al., (2021). All identified fungal genera are classified as storage fungi since they can proliferate under low humidity storge conditions (Jedidi et al., 2017).

Our results showed that maize samples were predominantly contaminated with *Cladosporium* and *Curvularia* species, while barley and sorghum samples contained various *Aspergillus* species. Notably, *Aspergillus* was not detected in any maize samples. The molecular identification through PCR amplification of the rDNA ITS region provided a powerful complementary approach to traditional morphological methods, allowing for precise species-level identification of the isolated fungi. This molecular characterization revealed five distinct species (*Aspergillus tubingensis, Aspergillus oryzae, Cladosporium oxysporum, Curvularia chonburiensis*, and *Curvularia lunata*) and demonstrated the superiority of DNA-based techniques over conventional microscopic methods for distinguishing between closely related fungal species that may have similar morphological characteristics but different mycotoxin-producing potentials. Maize samples also had lower relative abundance of 'other genera' compared to barley and sorghum samples. This difference may be attributed to the distinct environmental conditions of maize fields compared to barley or sorghum fields, with maize being more susceptible to fungal growth under higher temperatures and lower humidity. In addition, tocultivational conditions (temperature, precipitation, etc.) that vary from region to region, storage parameters such as temperature and humidity could also influence fungal proliferation. Similar findings have been reported by (Sultan, et al., (2022).

Most of the fungi and molds produce mycotoxins as secondary metabolites under favorable conditions such as high temperature and moisture, potentially contaminating food commodities, grains, and crops during both pre- and post-harvesting. (Kumar et al., 2021) Therefore, their identification and quantitative assessment of mycotoxin level are significantly important. Accurate mycotoxin quantification requires sophisticated sampling protocols, careful sample preparation, efficient extraction methods, and advanced and robust analytical techniques.

Food safety and security are fundamental consumer needs. While consumers can be exposed to contaminants through water consumption, inhalation, and skin contact, the most significant route of mycotoxins exposure is through ingestion of contaminated food, including cereals and animal-derived products. Mycotoxins present in animal feed can accumulate in animal tissues such as liver, kidneys, and can be transferred to (Wang et al., 2018). Consequently, all grain imported into Qatar undergo rigorous mycotoxin testing to ensure compliance with the country's food safety and security standards. Imported grain samples are also rigorously monitored for the presence of any

fungi under practical storage conditions. While the absence of fungi generally indicates minimal risk of contamination with mycotoxin, The detection of fungi suggests the potential for mycotoxin production, necessitating careful evaluation of such imported samples.

The grain samples analyzed in our work indicated the presence of three different fungal genres based on the traditional method of identification including the study of fungal morphology of purified fungal on potato dextrose agar and its microscopic identification. The isolated fungal genera were subjected to molecular identification and confirmed the presence of five species of fungi as listed in **Table 3**. All the samples were subjected to the quantitative estimation of mycotoxin level to ensure that the grains are free from any mycotoxin contamination.

Mycotoxins that exert their toxic effects on grains include aflatoxins (AF), deoxynivalenol (DON), fumonisins (FUM), zearalenone (ZEA), ochratoxin A (OTA), T2, HT-2, ergot alkaloids, and patulin (PAT). Isolated mycotoxins can be found in grain crops all over the world because the fungal species that produce them are widely distributed. (Chinaza Godseill Awuchiet al., 2022 and Alex Njugi Wangeci., 2018) Therefore, it is important to constantly monitor the level of mycotoxin contamination in all the imported grain samples to control huge economic losses of the country and to prevent the posed risks to animals and humans.

Based on the reoccurrence of fungal genera, three different samples from sorghum (sample-6), maize (sample-4) and barley (sample- 7) tested for aflatoxin B1, aflatoxin B2, aflatoxin G1, aflatoxin G2, ochratoxin A, Zearalenone, Deoxynivalenol, fumonisins B-1 or fumonisins B-2 had concentrations below the detection limits. Additionally, no Fusarium-derived mycotoxins or Trichothecenes (TCT) compounds were detected in any of the grain samples, confirming the country's food safety strategy for imported products.

Providing food availability, access to food, use of food, and stability to all people at levels that allow them to be physically and economically able to access affordable, safe, and nutritious food to keep them active and healthy is one of the main goals of every country. Food security is undermined when one of these goals weakens (Patchimaporn Udomkun et al., 2017 and A. O Adeyeye et al 2022). The state of Qatar has always prioritized food safety to provide the population with safe food that can cause no potential harm or danger to human health.

5. Conclusion

This study aimed to identify fungal species contaminating forage grains and to detect significant mycotoxins in grains imported into Qatar. Using both traditional morphological and molecular identification methods, we identified five different fungal species in sorghum, maize, and barley samples. The molecular identification through PCR amplification of the rDNA ITS region proved especially valuable, allowing for precise species-level identification that would not have been possible with conventional microscopic methods alone. This approach revealed five distinct species: *Aspergillus tubingensis*, *Aspergillus oryzae*, *Cladosporium oxysporum*, *Curvularia chonburiensis*, and *Curvularia lunata*, demonstrating the superiority of DNA-based techniques for distinguishing between closely related fungal species that may have different mycotoxin-producing potentials. All analyzed samples contained mycotoxin levels below detection limits, confirming that the imported grains meet Qatar's food safety standards. These findings support the effectiveness

of Qatar's current import monitoring systems in ensuring that forage grains entering the country are safe for animal consumption and pose no threat to the food chain.

Author Contributions

M.M; Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Supervision, Funding acquisition. H.A; Resources, Project administration. S.A; Validation, Resources. T.Y; Investigation, Data curation, Visualization. S.T; Methodology, Investigation. A.M; Methodology, Software, Formal analysis, Writing - review & editing. All authors have read and agreed to the published version of the manuscript.

Acknowledgment

This research study was made possible by UREP grant # UREP27-036-4-001 from the Qatar national research fund (a member of Qatar foundation).

5. Reference

A. O Adeyeye, T. J., Ashaolu, F., Idowu-Adebayo., 2022. Mycotoxins: Food Safety, Consumer Health and Africa's Food Security , Research Article, Polycyclic Aromatic Compounds, https://doi.org/10.1080/10406638.2021.1957952

Alex Njugi Wangeci., Maribel González-Villa., Jared Nyang'au., Stephen Ahenda. 2018, Aflatoxin Contamination in Maize Used as Animal Feed for Cattle and Its Potential Mitigation Measures, Current Situation In Kenya, *IOSR Journal of Environmental* Science, Toxicology and Food Technology (IOSR-JESTFT) e-ISSN: 2319-2402, p- ISSN: 2319-2399.Volume 12, Issue 7 Ver. I (July. 2018), PP 35-42, DOI: 10.9790/2402-1207013542.

Alkahtani, Muneera D. F., Mazen., M.M. El-Naggar, M. A. and Arfa, M. K. 2011. The Relationship Between Some Mycotoxins Excretion and Bean Seed Discoloration. Journal of Plant Sciences. 2011, 6(4),182-189.

Arlyn Ackerman , Anthony Wenndt , Richard Boyles., 2021. The Sorghum Grain Mold Disease Complex: Pathogens, Host Responses, and the Bioactive Metabolites at Play. Frontier in Plant Science, DOI: 10.3389/fpls.2021.660171.

Barnett, H.L., and Hunter, B.B.1972. The Illustrated Genera of Fungi. 3rd Edn., Burgess Publishing Company, Minnesota, Pages: 241.

Belkacem-Hanfi, N., Semmar, N., Perraud-Gaime, I., Guesmi, A., Cherni, M., Cherif, I., Boudabous, A. and Roussos, S. 2013. Spatio-temporal analysis of post- harvest moulds genera distribution on stored durum wheat cultivated in Tunisia. J. Stored Prod. Res. 55, 116–123.

Chinaza Godseill Awuchi, Erick Nyakundi Ondari, Sarah Nwozo, Grace Akinyi et al, Aspergillus derived mycotoxins in food and the environment: Prevalence, detection, and toxicity, Toxins (Basel). 2022 Mar; 14(3): 167.doi: 10.3390/toxins14030167

Gil-Serna, J., Mateo, E.M., González-Jaén, M.T., Jiménez, M., Vázquez, C. and Patiño, B.2013. Contamination of barley seeds with Fusarium species and their toxins in Spain: an integrated approach. Food Addit. Contam. 30 (2), 372–380.

Hocking, A.D., Pitt, J.I., Samson, R.A. and Thrane, U. (eds). 2006. Advances in Food Mycology. Springer Science Business Media, Inc., 233 Spring Street, New York, NY 10013, USA. 375 pp.

Ines Jedidi, Carlos Soldevilla, Amani Lahouar, Patricia Marín, MaríaTeresa González-Jaén, Salem Said., 2017. Mycoflora isolation and molecular characterization of Aspergillus and Fusarium species in Tunisian cereals. Saudi Journal of Biological Sciences. Volume 25, Issue 5, July 2018, Pages 868-874. https://doi.org/10.1016/j.sjbs.2017.11.050.

Jurado, M., Vázquez, C., Marín, S., Sanchis, V. and González-Jaén, M.T., 2006. PCR-based strategy to detect contamination with mycotoxigenic Fusarium species in maize. Syst. Appl. Microbiol. 29, 681–689.

Kehinde, M.T., Oluwafemi, F., Itoandon, E.E., Orji, F.A. and Ajay, O.I., 2014. Fungal profile and aflatoxin contamination in poultry feeds sold in Abeokuta, Ogun State, Nigeria. Nigerian Food Journal, 32(1), 73-79.

Krnjaja, V., Stojanović, A., Stanković, S., Lucić, M., Bijelić, Z., Mandić, V. and Mićić, N., 2017. Fungal contamination of maize grain samples with a special focus on toxigenic genera. Biotechnology in Animal Husbandry, 33(2),233-241.

Kumar, A., Pathak, H., Bhadauria, S. *et al.* (2021). Aflatoxin contamination in food crops: causes, detection, and management: a review. *Food Prod Process and Nutr* **3**, 17. https://doi.org/10.1186/s43014-021-00064-y

Leslie, J.F. and Summerell, B.A. 2006. The Fusarium Laboratory Manual. 1st Edn., Blackwell Publishing, Iowa, USA., ISBN: 0-8138-1919-9.

Mahato, D.K., Lee, K.E., Kamle, M., Devi, S., Dewangan, K.N., Kumar, P. and Kang, S.G., 2019. Aflatoxins in food and feed: An overview in prevalence, detection, and control strategies. Frontiers in Microbiology, 10, DOI: 10.3389/fmicb.2019.02266.

Marasas, W. F. O., Rabie, C. J., Lubben, A., Nelson, P. E., Toussoun, T. A., & Van Wyk, P. S. (1988). Fusarium nygamai from millet in southern Africa. *Mycologia*, *80*(2), 263-266.

Misihairabgwi, J. M., Ezekiel, C. N., Sulyok, M., Shephard, G. S., and Krska, R. (2017). Mycotoxin contamination of foods in Southern Africa: a 10-year review (2007-2016). *Crit. Rev. Food Sci. Nutr*. 58, 43–58. DOI: 10.1080/10408398.2017.1357003.

Moss, M.O., 1996. Mycotoxins. Centenary review. Mycol. Res. 100, 513–523.

Okun, D.O., Khamis, F.M., Muluvi, G.M., Ngeranwa, J.J., Ombura, F.O., Yongo, M.O. and Kenya, E.U., 2015. Distribution of indigenous strains of atoxigenic and toxigenic Aspergillus flavus and Aspergillus parasiticus in maize and peanuts agro-ecological zones of Kenya. Agriculture and Food Security, 4(1),2- 10.

Patchimaporn Udomkun, AlexanderNimo Wiredu, Marcus Nagle. *et al* (2017) Mycotoxins in Sub-Saharan Africa: Present situation, socio-economic impact, awareness, and outlook. Food Control. Food Control.8. Volume 72, Part A, Pages 110-122. https://doi.org/10.1016/j.foodcont.2016.07.039

Penagos-Tabares, F., Khiaosa-Ard, R., Nagl, V., Faas, J., Jenkins, T., Sulyok, M., & Zebeli, Q. (2021). Mycotoxins, phytoestrogens and other secondary metabolites in Austrian pastures: Occurrences, contamination levels and implications of geo-climatic factors. *Toxins, 13*(7), 460.

Pereira, C. S., Cunha, S. C. and Fernandes, J. O. 2019. Prevalent Mycotoxins in Animal Feed: Occurrence and Analytical Methods. Toxins 11(5): 62.

Reddy, K.R.N., S.B. Nurdijati and B. Salleh, 2010. An overview of plant-derived products on control of mycotoxigenic fungi and mycotoxins. Asian J. Plant Sci., 9: 126-133.

Roda F. Al-Thani., Bassam T. Yasseen. 2021, Microbial Ecology of Qatar, the Arabian Gulf: Possible Roles of Microorganisms, Sec. Aquatic Microbiology, Volume 8 - 2021 https://doi.org/10.3389/fmars.2021.697269.

Rodrigues, I. and Chin, L.J. 2012. A comprehensive survey on the occurrence of mycotoxins in maize dried distillers' grain and soluble sourced worldwide. *J. Wor. Myco.* **5**: 83–88.

Romina Alina Marc., 2022.Implications of Mycotoxins in Food Safety. Book edition on Mycotoxin and safety, DOI: 10.5772/intechopen.102495.

Sampietro, D.A., Marín, P., Iglesias, J., Presello, D.A., Vattuone, M.A., Catalan, C.A.N. and González-Jaén, M.T., 2010. A molecular based strategy for rapid diagnosis of toxigenic Fusarium species associated to cereal grains from Argentina. Fungal Biol. 114, 74–81.

Sardiñas, N., Gil-Serna, J., Santos, L., Ramos, A.J., González-Jaén, M.T., Patiño, B. and Vázquez, C., 2011. Detection of potentially mycotoxigenic Aspergillus species in Capsicum powder by a highly sensitive PCR- based detection method. Food Control 22 (8), 1363–1366.

Speijers, G.J. A. and Speijers, M.H.M. 2004. Combined toxic effects of mycotoxins. *Toxicol.Lett.* 153:91-98.

Sultan, Z., Nuhu, A.A., Aminu, I.M., Kamal, A.A. and Sumayya, **S.S.,** Isolation and Molecular Identification of Fungi Associated with Stored Grains Sold at Dawanau and Rimi Markets of Kano State, Nigeria. Acta Scientific AGRICULTURE (ISSN: 2581-365X). Volume 6 Issue 6 June 2022. DOI**:** 10.31080/ASAG.2022.06.1145.

Wang L et al. 2018 Occurrence and quantitative risk assessment of twelve mycotoxins in eggs and chicken tissues in China. Toxins; 10:47

White, T. J., Bruns, T., Lee, S. J. W. T., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications, 18(1), 315-322