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Molecular Procedures for Detection of Mycotoxegenic Fungi in Wheat Supplies in Saudi Arabia

Khalid S. Al-Zuaagi[1], Mohammed Abdo Moslem[2]*, Sarfaraz Hadi[2] and Mohammed Rabbani[2]

[1]Department of Central Sterile Services, Prince Sultan Military Hospital, Riyadh, Saudi Arabia.
[2]Department of Botany & Microbiology, College of Science, King Saud University, Riyadh, Saudi Arabia.

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Mycotoxins are secondary metabolites produced by molds growing on grain, feed, or food in the field or in storage. Some of these compounds may be seriously detrimental to human health. Mycotoxins of concern are: aflatoxin, vomitoxin, zearalenone, and fumonisin. Early detection of mycotoxins or the fungi producing them is crucial for preventing supply of contaminated food material including wheat grain. This study was undertaken to develop rapid, reliable and reproducible procedures for screening the wheat grain supplies being imported into Saudi Arabia. Pure cultures of mold species were isolated from the grain samples collected from discrete regions of Saudi Arabia. Enzymatic and antibacterial activity of the isolates was assayed to develop marker systems for detecting the molds in a sample. Major mycotoxins were detected and quantified with HPLC (high performance liquid chromatography) procedures. Mycotoxegenic mold species were identified by PCR (polymerase chain reaction) procedures using primers specific for ITS (internal transcribed spacers) and microsatellite sequences of the rDNA. The study showed that presence of mycotoxins or the molds producing them may be detected at an early stage in wheat grain samples with the help of molecular procedures.

Key words: Mycotoxins, Molds, Wheat grain.

Mycotoxins are secondary metabolites produced by molds such as *Aspergillus, Penicillium* and *Fusarium*. These hazardous compounds may enter the food chain in the field itself or during storage, especially when temperature and humidity are moderate to high favoring the growth of fungi[1]. Of the many mycotoxins, ochratoxinA (OTA) is clinically more important together with aflatoxins, fumonisins, trichothecenes and zearalenone. It is produced by *Penicillium verrucosum* in temperate or cold climates[2], by *Aspergillus* species, such as *A. ochraceus*, *A. alliaceus*[3, 4], *A. carbonarius*, *A. niger* and *A. melleus*[5, 6] in warmer and tropical parts of the world. This mycotoxin is nephrotoxic, carcinogenic, hepatotoxic, teratogenic and immunosuppressive[7].

Early detection of mycotoxins or the molds that may produce them is a crucial factor for monitoring and preventing the entry of contaminated food supplies into a designated area. This is, generally, a big challenge as food supplies are huge in size and fast in pace. It has been estimated that even where monitoring systems are in place, only 5-25% of imported food supplies are tested for mycotoxins[8]. Methods practiced in most places are time consuming, labor-intensive, costly, and require mycological expertise and facilities. Immunological methods and diagnostic media have limitation in identifying the aflatoxigenic fungi; since false positives are easy to come by and purification of the samples is a necessary prerequisite.

* To whom all correspondence should be addressed.
E-mail: mbmoslem@ksu.edu.sa

This study was undertaken to develop rapid, reliable and reproducible procedures for screening the wheat grain supplies being imported into Saudi Arabia. An extrapolative design was adopted for the study where findings of the experiments with the pure cultures could be corroborated with screening of the grain samples.

MATERIALS AND METHODS

Collection of wheat samples

Samples of wheat grains were collected from five different regions of the kingdom, namely, Riyadh, Jeddah, Dammam, Al-Qasim, and Hail. In each region, seed samples were collected randomly from flour mills at different locations and were pooled together to make a bulk of approximately 5 kg following the procedures of Yossef[9]. The samples were stored at 3-5 °C till their use for isolation of fungi.

Microscopic examination of the fungal isolates

Pure cultures of fungi were isolated from the grain samples by plating on modified Czapek Dox medium (Winlab Co., UK) according to the procedfure of Yossef[9], Bokhary[10], and Furlong *et al*.[11].

Isolates were stained with cotton blue-lactophenol and were examined under the microscope for tentative identification on the basis of morphological features. Identification was done with the procedures and the key in practice at the Mycology Center of Assuit University, Egypt.

Isolates apparently showing superior growth and mat forming ability were selected out and were consistently used for different experiments during the study.

Determination of enzyme activity

For cellulose (Cx) activity, the reaction mixture contained 2 ml of crude enzyme extract obtained from grains inoculated with known fungal isolate; 2 gm of Whatman No. 1 filter paper and 4 ml phosphate buffer solution (pH5.6). The mixture was incubated at 30 ºC for one hr. The activity of cellulase was measured as level of reducing sugars according to the method of Remero *et al*.[12].

For estimating polygalacturinase (PG), the reaction mixture consisted of 2 ml of pectin culture filtrate and 4ml of 1.2 % pure pectin in phosphate buffer (pH 5.5). The mixture was incubated at 30 ºC for one hour, then the reaction was stopped by boiling in a water bath for 15 min. The mixture without crude enzyme was used as a control. The enzyme activity was measured according to the method proposed by Thomas and Dutcher[43].

For pectin methyl esterase (PME), the reaction mixture contained 2.5 ml of crude enzyme extract and 15 ml of 1.2 % pure pectin in phosphate buffer (pH 5.5) then the reaction was stopped by boiling in a water bath for 15 min. The mixture without crude enzyme was used as a control. The enzyme activity was measured according to the method proposed by Thomas and Dutcher[43].

Mycotoxin bioassay using two different species of bacteria

Bacillus subtillus and *B. megaterium* are considered indicator strains against mycotoxins[13]. The cultures were obtained from Bacterial Disease Dept. of Plant Pathology Research Institute, Cairo, Egypt. The cultures were grown in tryptone-glucose-yeast extract broth (TGY). Tubes were inoculated with (1.1×10^{-4} g dry weight ml^{-1}) of exponentially growing cells followed by incubation at 37 °C for 24 hr.

Known fungal isolates were grown on rice as the substrate for aflatoxin production, according to procedure of Shotwell *et al*.[13]. Mycotoxin was extracted by grinding the grains and dissolving the contents in methanol. The solvent was evaporated under vacuum and the residue was passed through 2.2 µm filter to remove fungal spores.

Sterilized filter-paper discs (0.6 cm diameters) were soaked in fungal extract for1 hr and then were transferred to petri plates containing TGY agar with diagnostic bacteria. Three replicate tests were conducted for each isolate. Sterilized distilled water was used as a control. Incubation of 48 hr at 37°C was provided to each test plate. At the end of incubation, inhibition zones were measured for each fungal isolate. Average of the diameters for an isolate was recorded according to Tiwari *et al*.[14].

Estimation of mycotoxins using HPLC

Penicillium toxins

Known fungal isolates were grown on sterilized malt extract medium in 100 ml flasks for 7-10 days at 27 ± 2ºC with three replicates per isolate. The method described by Christian[15] was used to determine patulin. After the incubation, cultures

were blended for 2 min using a high speed homogenizer and filtered using glass filter paper. patulin was extracted from homogenized filtrate using acetonitrile: water (5:95 v:v) solution (liquid mobile phase).

The solvent was then evaporated at 35°C under vacuum. The dried residues containing patulin were dissolved in 1 ml of the same liquid mobile phase solution. The extract was passed through a 0.45 µm filter. Analysis of compounds was performed on an HPLC machine (PerkinElmer® Brownlee™) using a C18, 250 mm column. The instrument was equipped with UV detector with reading wave length set at 280 nm. Total run time for the separation was approximately 25 min at a flow rate of 1 ml min^{-1}.

Quantitative analytical method described by Stubblefield *et al.*[16] was used for citreoviridin determination. The toxin was extracted with dichloromethane, and the extract was partially purified on silica and amino solid-phase extraction (SPE) columns. The extract was analyzed for citreoviridin by normal-phase liquid chromatography, using a mobile phase of ethyl acetate: hexane (75:25) at a flow rate of 1.5 ml min^{-1} with a fluorescence detector to measure the yellow fluorescence at 388 nm excitation and 480 nm emission.

***Aspergillus* toxins (Aflatoxins)**

Isolates were grown on sterilized SMKY liquid medium containing sucrose 20g l^{-1}, magnesium sulfate 0.5g l^{-1}, potassium nitrate 3g l^{-1}, and yeast extract 7g l^{-1} [17] for 10 days at 27 ±2ºC with three replicates per isolate. Cultures were blended for 2 min using a high speed homogenizer and filtered though Whatman's filter paper. Aflatoxins were extracted from the homogenized filtrates using methanol (80:20 methanol/filtrate). Solvent was evaporated under vacuum at 35ºC; and dried residue containing aflatoxin was dissolved in 1 ml of the liquid mobile phase containing methanol: acetic acid: water (20/20/60).The solution was stored in dark vials.

HPLC procedure suggested by Christian[15] was used to detect and estimate aflatoxin level. The extract was passed through a 0.45 µm filter. Analysis of compounds was performed on HPLC (PerkinElmer® Brownlee™) using a C18, 100 mm × 4.6 mm, 3µ column. Reading wavelength of 365 nm was used to detect the toxin. A total run time of approximately 25 min at a flow rate of 1 ml min^{-1} was used for separation.

***Fusarium* toxins**

Fumonisin toxins were determined according to the method described by Mazzani *et al.*[18]. Isolates were grown on sterilized SMKY liquid medium for 10 days at 27 ±2ºC with three replicates per isolate. Fungal culture of each flask was blended with 5 gm sodium chloride and 100 ml of methanol: water (80:20) solution at a high speed for one min. The slurry was filtered through glass-fiber filter 2.2µm. Ten ml of the filtrate was diluted with 40 ml of wash buffer and filtered again through 1 µm micro-fiber filter. Ten ml of the diluted extract was passed through a fumontest column (Vicam Co) and the column was washed using 10 ml of the same dilute solution. Fumonisin was eluted by passing one ml of HPLC grade methanol through the column and then elutes were re-collected. One ml of each, developer-A (Vicam product No.G5005) and developer-B (Vicam product No. G5004) were added to the elutant and placed in a calibrated fluorometer (Series-4 / Vicam).

Zearalenone and vomitoxin concentration was determined as mentioned for fumonisin toxin, but the dilution was made with 49 ml distilled water which was passed through a Zearatest and/or vomitoxin column (Vicam Company) and then measured in calibrated fluorometer (Series-4/Vicam).

Polymerase chain reaction (PCR) analysis

Genomic DNA was extracted from fungal isolates using a modified sodium dodecyl sulphate (SDS) extraction procedure[42]. PCR analysis was performed using primers targeting internal transcribed spacer (ITS) and microsatellite (simple sequence repeats – SSR) sequences.

ITS-PCR was carried out in 25-µl reaction volume. Three µl of DNA template (1ng μl^{-1} quantified with a spectrophotometer) was added to a 5 µl master mix (Taq Master/high yield - Jena Bioscience GmbH) containing all the reagents required for PCR (Thermostable DNA polymerase, dATP, dCTP, dGTP, dTTP, $(NH_4)_2SO4$, $MgCl_2$ and Triton X-100) except template and primer. To this mix 13µl of PCR grade water (Jena Bioscience GmbH), 2µl of 10 pmol ITS4 primer and 2 µl of 10 pmol ITS3 primer was added. The sequence of ITS3 primer was: 5'-TCCTCCGCTTATTGATATGC-3' and the sequences of ITS4 primer was: 5'-TCC TCC GCT TAT TGA TAT GC-3' [19]. Primers were procured

from GE Healthcare. Reactions were performed in a thermal cycler (Techne TC-312, Techne, Stone, UK) programmed for following cycling parameters: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. A final extension at 72°C for 10 min was provided at the end of the reaction. PCR products were separated on 1.5% agarose gel containing 0.05 µg ml^{-1} ethidium bromide, using 5µl of each sample + 3µl gel loading dye + 3µl ultrapure water. Each well was loaded with 10µl of the mixture. Amplification products on the gel were visualized under UV radiation in UVI Bandmap hood (UVTec, Cambridge).

Same procedure was used for microsatellite PCR except that SSR-specific primers were used at the rate of 2µl of 20 pmol concentration per reaction. Sequences of the primers were: primer-1 [(CAG)5] CAG CAG CAG CAG CAG; primer-2 [(GTG)5] GTG GTG GTG GTG GTG, primer-3 [T3B] 5' -AGG TCG CGG GTT CGA ATC C-3' , and primer-4 [M13] 5' -GAGGGTGGCGGTTCT-3' (Metabion International AG GmbH). Annealing temperature was set at 50°C to suit the melting temperature of the primers.

RESULTS AND DISCUSSION

In all, 70 isolates belonging to 8 genera were purified from 120 wheat samples collected from different regions of Saudi Arabia. Microscopic

Table 1. Enzymatic activity of selected isolates derived from wheat grain samples

Fungus	Isolate No.	Pectinase activity (Uml^{-1})		Cellulase (cx) activity (CMC Uml^{-1})	Lipase activity (shaken) (Uml^{-1})	α- amylase activity (Uml^{-1})
		PG[1]	PME[2]			
Penicillium	51	0.80	0.92	0.37	61	2.90
	57	1.26	1.41	0.42	73	3.00
	59	1.18	1.33	0.42	72	3.17
	60	0.83	0.95	0.34	59	3.38
	63	0.89	1.00	0.36	57	3.99
	81	1.15	1.29	0.43	70	3.37
	89	0.91	1.10	0.36	55	3.01
	90	1.45	1.62	0.59	86	2.84
	91	0.83	0.93	0.35	54	0.60
	95	1.20	1.34	0.59	89	3.80
	96	0.84	0.94	0.32	53	4.00
	99	0.89	1.00	0.33	59	2.10
Aspergillus	30	0.79	0.88	0.39	92	1.91
	32	1.24	1.39	0.45	107	3.32
	33	0.78	0.87	0.39	90	3.99
	39	2.05	2.30	0.65	120	2.97
	47	0.94	1.05	0.38	89	1.68
	49	1.65	1.85	0.64	116	2.10
	54	1.21	1.36	0.45	108	3.10
	73	1.17	1.30	0.46	107	3.80
	74	1.75	1.96	0.64	118	4.00
	78	1.89	2.10	0.64	115	3.70
	82	1.10	1.23	0.47	106	3.30
	92	0.79	0.85	0.37	94	
Fusarium	56	1.22	1.35	0.45	61	1.60
	83	1.85	2.07	0.63	69	1.59
	93	1.12	1.25	0.44	65	1.75
	94	1.93	2.15	0.63	70	1.89

1 = Polygalacturinase; 2 = Pectin methyl esterase

examination showed that out of these isolates, 29(41%) belonged to *Pencillum* species, 28 (40%) to *Aspergillus* species, 4(5%) to *Fusarium* species, 3(4%) to *Rhizopus* species, 3(4%) to *Eurotium* species, 1(1%) to *Mucor* species, 1(1%) to *Nigerospora* species, and 1(1%) to *Arthrinium* species. *Penicillium chrysogenum, Aspergillus flavus,* and *Fusarium semitectum* were the most prevalent species of their respective genera.

Enzymatic activity of selected isolates

All the tested isolates showed pectinolytic, cellulytic, lipolytic and amylolytic activity (Table-1). Enzymatic activity of the isolates varied significantly between and within the fungal genera. In addition, differences in activity of the studied enzymes were observed between the isolates of the same species also. The highest values for polygalacturonase (2.05 U ml^{-1}), pectin methyl esterase (2.30 U ml^{-1}), cellulose (0.65 U ml^{-1}) and lipase (120 U ml^{-1}) activity were shown by a single isolate (No. 39) of *Aspergillus*. Maximum amylase activity (4.00 U ml^{-1}) was shown by isolates of *Penicillium* (No. 96) and *Aspergillus* (No. 74). *Fusarium* isolates generally showed moderate level of enzyme activity.

Many molds including *Aspergillus*, *Fusarium* and *Penicellium* are known to produce cellulases and other enzymes[20, 21, 22]. *A. niger* and *P. citrinum* are reported to produce amylase, protease and lipase in fruit drink[23]. While some times enzyme producing capacity of the fungi is used for human benefit, in most of the cases fungal enzyme production on the substrate leads to quality degradation and spoilage of the food material.

Table 2. Bioassay for mycotoxin production by selected fungal isolates using two species of bacteria

Fungus	Isolate No	Average inhibition zone (mm)	
		Bacillus subtillus	*Bacillus megatherium*
Penicillium	51	0.0	0.0
	57	4.4	6.5
	59	6.5	9.5
	60	0.0	0.0
	63	0.0	0.0
	81	0.0	0.0
	89	0.0	0.0
	90	8.7	12.5
	91	0.0	0.0
	95	11.5	16.5
	96	0.0	0.2
	99	0.0	0.0
Aspergillus	30	0.0	0.0
	32	4.9	7.0
	33	0.0	0.0
	39	13.5	19.0
	47	0.2	0.0
	49	8.0	11.5
	54	4.5	6.0
	73	3.5	4.5
	74	9.0	13.0
	78	9.5	14.0
	82	5.0	7.0
	92	0.0	0.0
Fusarium	56	4.0	5.5
	83	10.5	15.5
	93	6.0	8.5
	94	9.5	14.0

Enzyme activity recorded in wheat samples in the present study is also likely to cause deterioration in quality of the grain. Wheat is consumed mainly for its starch. Amylase activity of all the three major fungi observed in this study means that the grain would lose some of its starch resulting in reduced nutrition. Similarly, pectin and cellulose are major quality attributes of wheat flour. The former is essential for dough quality while latter provides the much needed roughage in the wheat flour-based foods. Liberation of pectinases, like polygalcturonase (PG) and pectin methyl esterase (PME), and cellulase by the contaminating fungi in the wheat samples highlights the probability of quality deterioration of grain due to these enzymes. Wang *et al.*[24] have also reported quality deterioration of wheat due to activity of *Fusarium* sp. Impact of enzymes on the grain quality notwithstanding, their presence in the samples would give an indication of the contamination of wheat samples with active molds.

Bioassay for mycotoxin production

In the case of *Penicillium*, out of 12 isolates tested, 4 formed inhibition zone against

Table 3. Estimation of mycotoxins produced by selected fungal isolates using HPLC

Fungus	Isolate No.	Type of mycotoxin			
Penicillium		Patulin (ppb)	Citreoviridin (ppb)		
	51	-	-		
	57	65.0	25.0		
	59	49.5	32.5		
	60	-	-		
	63	-	-		
	81	31.5	20.0		
	89	-	-		
	90	79.5	45.5		
	91	-	-		
	95	65.5	53.0		
	96	-	-		
	99	-	-		
Aspergillus		Aflatoxins (ppb)			
		B1	B_2	G_1	G_2
	30	8.5	0.0	0.0	0.0
	32	42.5	9.8	0.0	9.0
	33	0.0	0.0	0.0	0.0
	39	109.0	20.1	35.1	16.5
	47	0.0	0.0	0.0	0.0
	49	85.5	22.5	26.4	12.3
	54	39.5	10.6	20.2	8.1
	73	60.4	11.2	21.9	9.7
	74	77.5	18.4	27.8	14.8
	78	74.0	18.1	24.7	6.4
	82	45.5	10.8	14.6	0.0
	92	10.8	0.0	0.0	0.0
Fusarium		Zearalenone (ppb)	Vomitoxin (ppb)	Fumonisin (ppb)	-
	56	165	535	1175	-
	83	255	765	1530	-
	93	190	695	1280	-
	94	280	826	1694	-

Bacillus subtillus and 5 against *Bacillus megatherium* (Table 2). Largest zone was of 11.5 mm against former and 12.5 mm against later bacterium. Nine *Aspergillus* isolates out of 12 tested developed inhibition zones against *B. subtillus* with the largest diameter of 9.5 mm and 8 isolates showed antagonism against *B. megatherium* with the largest diameter of 19.0 mm. All the four tested isolates of *Fusarium* developed inhibition zones against both the bacteria with zones up to 15.5 mm in diameter.

Presence of mycotoxins in most of the samples could be detected with the help of these assays. The tests are simple, quick and less expensive and provide a provisional indication of the presence of mycotoxins in a sample. Similar and many other types of bioassays based on chemical markers are in practice for detection of mycotoxins and presence of mycotoxigenic fungi in food and feed samples[25,26,27]. Successful application of these tests for detection of mycotoxigenic fungi in wheat sample in this study is an encouraging sign of the scope of their application in a contamination monitoring system in Saudi Arabia.

Estimation of mycotoxins using HPLC

Production of substantial level of mycotoxins could be detected in most of the isolates of *Penicillium, Aspergillus*, and *Fusarium* by HPLC procedures. In the case of *Penicillium*, 5 out of 12 isolates tested, produced patulin as well as citreoviridin (Table 3). In the case of *Aspergillus* 10 out of 12 tested isolates produced mycotoxins of class B_1, B_2, G_1, and G_2. Similarly all 4 isolates of

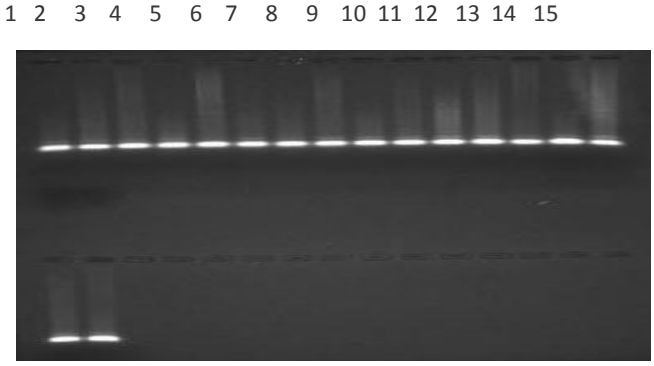

Fig. 1. ITS-PCR of selected fungal isolates using primers ITS4 (lanes 1-7: *Penicillium;* 8-16: *Aspergillus;* 17: *Fusarium*)

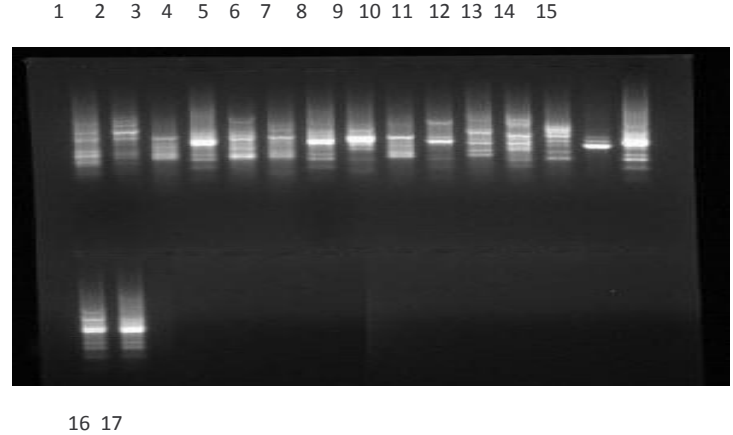

Fig. 2. Microsatellite PCR of selected fungal isolates using primer (CAG)5 (lanes 1-7: *Penicillium;* 8-16: *Aspergillus;* 17: *Fusarium*)

Fusarium produced zearalenone, vomitoxin, and fumonisin which are highly potent toxins.

Level of aflatoxin production was widely variable in *Aspergillus* isolates. Isolates 33 and 47 failed to produce any detectable amount of aflatoxins, and isolates 30 and 92 produced only B1aflatoxin. Isolate 39 was the highest producer of aflatoxin B_1, G_1 and G_2, since it produced 109.0, 35.1 and 16.0 ppb of these toxins respectively. Isolate 49 was the highest producer (22.5ppb) of aflatoxin B_2.

All examined *Fusarium* isolates were found to be toxin producers in HPLC procedures. In the case of *Fusarium* also, mycotoxin productivity varied between and within tested isolates. Isolate 94 was the highest producer of all kinds of *Fusarium* toxins. It produced 280, 826 and 1694 ppb of zearalenone, vomitoxin, and fumonisin respectively. The highest productivity of all isolates was for fumonisin followed by vomitoxin and zearalenone.

High performance liquid chromatography is a very potent tool for detection of small amount of mycotoxins in food and feed samples[28, 29]. We have been able to detect mycotoxins like, patulin and citreoviridin from *Penicillium,* aflatoxins from *Aspergillus*, and zearalenone, vomitoxin and fuminisin from *Fusarium* at ppb levels. These findings are in agreement with results of other workers. Cho *et al*.[30] have demonstrated effective use of HPLC for detection of patulin in juices. Tangi and pussmier[31], using HPLC, detected several mycotoxins including citreoviridin, patulin, and zearalenone in dust samples from grain storage bins. Levels of mycotoxins detected by them are comparable with our estimates. Maria *et al*.[32] also recovered similar levels of aflatoxins (B_1, B_2, G_1, and G_2) from samples of different spices. Yazar and Omurtag[33] have outlined HPLC procedures for determining zearalenone, fumonisin, and trichthecene contamination in cereal samples. Outcome of our HPLC studies suggests that besides PCR-based procedures, HPLC may also be used effectively for detection and quantification of mycotoxins in food grain samples in Saudi Arabia.

PCR analysis

DNA amplification by ITS-PCR using primers ITS3 and ITS4 showed amplification of the targeted sequences in all the tested isolates (Fig-1). This is an encouraging indication that primers used in the study can detect the presence of these fungi in a grain sample. Potential of ITS region for molecular characterization of various fungi has been well highlighted and utilized by Gomes *et al*.[34]. Kulik[35] has reported simultaneous and authentic detection of *Fusarium poae* and *F. sporotrichioides* strains from wheat samples using specific ITS primers. Boysen *et al*.[36] used ITS primers specific to 5.8S rDNA sequences to detect and identify *Penicillium* strains in animal feed. Such reports vindicate the approach of using ITS-PCR for detection and identification of mycotoxigenic fungi contaminating wheat supplies.

Microsatellite PCR (MP)

Using the microsatellite primers (CAG)5, (GTG)5, T3B, and M13 marked polymorphism was generated between and within the isolates of different species as shown in a representative image of the gel (Fig-2). This demonstrates the potential of the procedure not only for detection but also, marker based identification of the fungus contaminating a grain sample. Applicability and authenticity of using microsatellite PCR for detection and identification of fungal genotypes in diverse type of food material has been demonstrated in a large number of reports in the literature. Yin *et al*.[37] characterized toxigenic and atoxigenic isolates of *Aspergillus flavus* using primers targeting microsatellite region of the fungi in peanut samples. Microsatellite-based PCR has been extensively used for molecular characterization of *Aspergillus fumigates*[38] and *A. niger*[39]. Similarly, *Fusarium germinearum*[40] and *Penicillium*[41] strains have been authentically discriminated using microsatellite PCR.

This study demonstrates that molecular procedures involving HPLC and PCR can be extrapolated to detect the presence of mycotoxins or fungi producing them in wheat supplies entering markets in Saudi Arabia from different sources.

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