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Fungal contamination spices from Indonesia with emphasis on *Aspergillus flavus*

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Abstract: Filamentous fungi were isolated from ten spices collected from markets in Indonesia. The aim was to enumerate fungal contamination and to determine the toxigenicity of *Aspergillus flavus* strains on each of the spices. Viable fungal populations were determined using a dilution method. Toxigenicity of *Aspergillus flavus* was determined by culture on a quick screening coconut agar and by PCR using four sets of primers specific for aflatoxin pathway genes. All the tested spices were contaminated by storage fungi, Species of *Aspergillus* was the most commonly isolated moulds followed by species of *Fusarium*, *Mucor*, *Penicillium* and *Rhizopus*. The greatest number of *Aspergillus flavus* isolates were found on white pepper, followed by nutmeg, cardamom, and black pepper. The greatest number of *Aspergillus chevalieri* isolates were found on coriander, followed by nutmeg. Fifty strains of *A. flavus* were isolated, all of the strains produced large sclerotia and biseriate conidiophores. Their toxigenicity was assayed by the presence of yellow pigment on a quick screening coconut agar medium and PCR amplification of regulatory and structural genes in the aflatoxin pathway.

Keywords: aflatoxigenicity; aflatoxin pathway; *Aspergillus flavus*; fungal population; genes; spices

Aromatic spices are commonly produced in tropical countries. Most of the spices are used as ingredients in traditional foods or medicines or and as food supplements. Common dried spices such as nutmeg, pepper, cloves and cinnamon are widely cultivated and handled conventionally by subsistence farmers for domestic consumption. As is true for many agriculture products, dried spices are susceptible to microbial contamination during pre and post-harvest processing (Škrinjar *et al*. 2012; Toma & Abdulla 2013). During harvesting, distribution and storage, the temperature and relative humidity (RH) are beyond the control of most farmers. The spices are usually sun dried in the open, which also can lead to microbial contamination, especially by moulds (Dharmaputra *et al*. 2015). High fungal count on dried spices was reported by Hashem and Alamri (2010) and and Abdulla (2013). *Aspergillus*, *Penicillium* and *Rhizopus* were the most common moulds genera found. The presence of fungi on spices

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not only deteriorates the commodity and leads to loss of spice flavour but can cause mycotoxin contamination as well. The degree of spoilage and aflatoxin production by toxigenic *Aspergillus flavus* depend upon fungal strains, substrate, geographic area, climate, and plant culture system technique (Perrone *et al*. 2014). Strains of *Aspergillus flavus* are found in temperate and tropical regions in soil and agricultural areas with diverse strains and consisted of aflatoxigenic and non-aflatoxigenic (Ehrlich 2014).

A quick screening method for detecting putative aflatoxigenic strains of *Aspergillus flavus* relies on the presence of a yellow pigment in coconut agar medium (Lin & Dianese 1976; Davis *et al*. 1987). A more reliable method for differentiating aflatoxin-producing and non-producing strains utilizes PCR amplification of *aflR, ver*-1, *nor*-1 and *omt*-1 genes from the aflatoxin biosynthesis pathway Criseo *et al*. (2001). Erami *et al*. (2007) also applied polymerase chain reaction (PCR) using primer *aflR*, *nor*-1, *ver*-1, and *omt*-1 to detect the presence of regulatory (*aflR*) and structural genes (*nor*-1, *ver*-1, *omt*-1) that are involved in the aflatoxin biosynthesis pathway. The present study aimed to enumerate levels of fungal contamination and to determine toxigenic and non-toxigenic *A. flavus* strains in 10 kinds of store-dried spices collected from retailers at traditional markets in Medan, North Sumatera, Indonesia.

MATERIAL AND METHODS

Sample collection. Composite samples (45 g) containing ten kinds of store-dried spices (black pepper, candle nut, cinnamon, cloves, coriander, cardamom, cumin, nutmeg, star anise, and white pepper) were collected from nine retailers at five different traditional markets in Medan, North Sumatera, Indonesia from April to July 2018. Each spice sample (200 g/sample) was collected in a sterilized polyethylene bag and stored at –4°C until further use.

Sampling method to obtain a working sample. Each store-dried spice derived from each traditional market was analysed according to Dharmaputra *et al*. (2015). Every 200 g of spice was ground for 30 s (25 000 rpm) using Mill Powder Tech Model RT-04 (Mill Powder Tech Co., Taiwan). The finely powdered spice was then divided into sub-samples for downstream assays.

Determination moisture content. The moisture content of each spice was determined using the oven drying method (BSI 1980). Each of the ground spice (40 g) was dried in an oven at 130°C for 2 h with three replicates per sample.

Enumeration fungal population. Fungal population from each sample were enumerated, isolated, and identified using a serial dilution method on dichloran 18% glycerol agar (DG18) plate (Pitt & Hocking 2009). For each finely powdered spice, 25 g was placed in a 500 ml conical flask and diluted with 250 ml of sterilized distilled water. The suspension was shaken at 250 rpm for 2 min and one ml of the aliquot was poured with DG18 medium onto a Petri dish (9 cm diameter). Four dilutions (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) were made for each sample with three replicates of each. All of the plates were incubated for seven days at ambient temperature (29 ± 2°C). Separate fungal colonies were enumerated as colony forming unit (CFU/g) per sample (Dharmaputra *et al*. 2015). Individual colonies were sub-cultured on Czapek yeast extract agar (CYA) or CYA+20% sucrose (CYA20S) and identified morphologically according to Pitt and Hocking (2009).

Isolation of *A. flavus*. Each separate colony of *A. flavus* strain was transferred onto a Petridish (9 cm diameter) containing potato dextrose agar plates (PDA; Oxoid Ltd, UK) and incubated for seven days at ambient temperature (29 ± 2°C). The colonies then were sub-cultured again on PDA slants.

Sclerotial production of A. flavus. Sclerotia were separated from fungal colonies and media according to the procedure described by Novas and Cabral (2002) with modifications. Each *A. flavus* strain was cultured in a Petri dish (9 cm diameter) containing PDA (Oxoid Ltd, UK). Each plate (triplicates for each strain) was incubated for 14 days at 29 ± 2°C. Next, 10 ml of 0.05% Tween 80 was added onto the surface of the colony. Sclerotia were harvested using a small sterilized brush over a filter paper No. 2 (Whatman, UK). The fresh-dried harvested sclerotia were then counted.

Quick screening for A. flavus aflatoxigenicity on coconut agar. To determination the toxigenicity of *A. flavus*, each isolate was cultured on coconut agar medium (CAM) according to Lin and Dianese (1976). Briefly, 100 ml of coconut cream extracted from freshly shredded coconut endosperm was mixed with 36 g bacto agar and 900 ml distilled water). The pH medium was adjusted into 7.0 using 2 N NaOH and sterilized for 20 min at 120°C. A small hyphal fragment of each strain of *A. flavus* was inoculated onto the centre of a CAM plate in a Petri dish (9 cm diameter) and incubated for five days at ambient tem-

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perature (29 ± 2°C). The presence of yellow pigment on the reverse side of each plate indicated toxigenic strains. The presence or absence blue fluorescence surrounding the growing colony was examined using long-wave (365 nm) UV light and expressed as positive or negative toxigenicity (Davis *et al.* 1987).

Isolation of A. flavus DNA. To obtain mycelia for DNA extraction, each strains of *A. flavus* was cultured on a PDA plate for two days at 29°C. About 40 mg of the mycelia in a clean tube containing 600 µl nuclei lysis solution were ground using a micropestle. DNA extraction was conducted according to Mini Kit (Promega, USA) procedures. The homogenized mycelia were incubated at 65°C for 15 min and cooled at ± 29–30°C for 5 minutes. Two hundred microliter of a protein precipitation solution was added, stirred for 5 s and centrifuged at 13 000 g for 3 minutes. The supernatant was removed into a clean tube containing 600 µl isopropanol and then was centrifuged at 13 000 *g* for 2 minutes. The supernatant was decanted and resuspended with 600 l of 70% ethanol and centrifuged at 13 000 g for 2 minutes. Next, the rest of the remaining ethanol was aspirated. The air-dried pellet containing the DNA was resuspended with 50 µl of DNA rehydration solution and then vortexed for 5 seconds. One half microliter of RNAse solution was added and the samples were incubated at 37°C for 15 minutes. The DNA was then rehydrated at 65°C for 1 h or overnight at 4°C. The DNA concentration was determined using nanophotometer (No. 6042; IMPLEN, Germany). Subsequently, the DNA was examined by agarose gel (1%) electrophoresis (SCIE-PLAS Ltd, UK), stained with 0.1 mg/l ethidium bromide. The gel was visualized using Gel Doc (No. 13 200263; Uvitec, UK) under UV light (303 nm).

Identification of toxigenic A. flavus. Toxigenic and non-toxigenic *A. flavus* strains were identified using the quadruplex-PCR method developed by Criseo *et al.* (2001). The GeneAmp PCR Labcycler Gradient System (No. 1123280105; Sensoquest, Germany) was used to amplify regulatory (*aflR*) and structural genes (*nor*-1, *ver*-1, *omt*-1) with fragments sized 1032, 400, 895, and 1232 bp respectively. Four pairs of specific primers were employed to amplify the regulatory and structural genes as shown in Table 1.

The amplification mixture consisted of 12.5 µl PCR Mix, 2.5 µl of 10 µM F and R primer each, 2.5 µl nuclease free water and 5 µl DNA template for a final reaction volume of 25 µl. Amplifications were conducted with the following cycling: initial denaturation at 94°C for 10 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 2 min, extension at 72°C for 2 min and final extension at 72°C for 7 minutes. A 10 µl aliquot of the PCR products used for electrophoresis (SCIE-PLAS, UK) on a 1.2% agarose, gel stained with ethidium bromide (0.1 mg/l) and photographed under UV light (303 nm) (No. 13 200263; Uvitec, England) using a 100 bp marker ladder as a standard.

RESULTS AND DISCUSSION

A total of three genera representing 17 species of filamentous fungi were isolated from 10 different kinds of store-dried spices collected at retailers in traditional markets (Table 2). All of the spices were contaminated by filamentous fungi. Species of *Aspergillus* were the predominant moulds isolated and were found on each spice. Among the ten spices assayed, the highest number of fungi were isolated from black pepper followed by cardamom, nutmeg and white pepper, whereas, the lowest numbers were found from coriander, cumin, candlenut, star anise, cinnamon and cloves. *Fusarium* sp., *Mucor* sp. and *Rhizopus stolonifer* were isolated only from black pepper and nutmeg kernels.

Table 1. Primers, target gene, sequence PCR product size

Primer code	Gene	Primer sequence	PCR product size (bp)
aflR-F	*aflR*	5'-TATCTCCCCCCGGGCATCTCCCGG-3'	1032
aflR-R		5'-CCGTCAGACAGCCACTGGACACGG-3'	
nor-1-*F*	*nor*-1	5'-ACCGCTACGCCGGCACTCTCGGCAC-3'	400
nor-1-*R*		5'-GTTGGCCGCCAGCTTCGACACTCCG-3'	
ver-1-*F*	*ver*-1	5'-TGTCGGATAATCACCGTTTAGATGGC-3'	895
ver-1-*R*		5'-CGAAAAGCGCCACCATCCACCCCAATG-3'	
omt-1-*F*	*omt*-1	5'-GGCCCGGTTCCTTGGCTCCTAAGC-3'	1232
omt-1-*R*		5'-CGCCCCAGTGAGACCCTTCCTCG-3'	

https://doi.org/10.17221/18/2019-CJFS

Table 2. Fungal population of stored-dried spices

Fungal species	Fungal population (CFU/g)									
	black pepper[b] (10^3)	candle nut[b] (10^3)	cinnamon[a] (10^4)	cloves[b] (10^4)	coriander[b] (10^3)	cardamom[a] (10^3)	cumin[a] (10^3)	nutmeg[a] (10^3)	star annise[a] (10^3)	white pepper[b] (10^3)
Aspergillus candidus	1.3	0	0	0	0.5	0	0	0.9	0	10.7
A. carbonarius	0	0	0.1	0.1	0	0	0	0	0	0
A. flavus	0.3	0.1	0.1	0.1	0.1	0.6	0.1	0.9	0	22.6
A. fischerianus	0	0	0.1	0	0	0	0	0	0	0
A. fumigatus	5.4	0.1	0	0	0.2	2.2	0.1	0	0.1	0
A. niger	6.2	0.1	0	0	0	3.7	0	4.3	0	1.0
A. oryzae	0.1	0	0	0	0	0	0	0	0	0
A. restrictus	0	0	0	0	0	3.1	0	0	0	0
A. sydowii	4.9	0.3	0	0.1	0	0.6	0.5	5.7	0	71.6
A. tamarii	0.1	0.1	0	0	0	3.5	0	2.3	0	0.3
A. terreus	0.1	0	0	0	0	0.1	0	0	0	0.1
A. wentii	0	0.3	0	0	0	0	0	0.5	0	0
A. westerdijkiae	0	0	0	0	0	7.7	0.3	0	0	0
Eurotium chevalieri	3.8	2.8	0.9	0.1	38.2	4.6	1.1	26	0.1	10.6
E. repens		0	0	0	0	0.6	0.3	0	0	0
Fusarium sp.	0.1	0	0	0	0	0	0	0.4	0	0
Mucor sp.	0.4	0	0	0	0	0	0	0	0	0
Penicillium citrinum		0	0	0	0	0	0	3.0	0	0
Penicillium sp.	0.1	0.2	0	0.1	3.1	0.1	0.3	0	0.1	4.8
Rhizopus stolonifer	0	0	0	0	0	0	0	0.1	0	0

CFU – colony forming unit; [a]n = 44; [b]n = 45

As shown in Table 2, *Aspergillus chevalieri* (previously called *Eurotium chevalieri*) was the most common species isolated on all of the spices studied. The great number of *A. chevalieri* were observed from coriander (38.2×10^3 CFU/g) followed by nutmeg kernels (26×10^3 CFU/g). *A. chevalieri* is a common spoilage organism found on nuts and seeds (Samson *et al*. 2004); cinnamon, marjoram, cloves, and caraway (Dimić *et al*. 2008); and nutmeg (Dharmaputra *et al*. 2015; Nurtjahja *et al*. 2016).

Except for star annise, all spices were contaminated by *A. flavus*. The highest number of *A. flavus* isolates were found from white pepper followed by nutmeg kernel, cardamom and black pepper with counts of 22.6×10^3, 0.9×10^3, 0.6×10^3, and 0.3×10^3 CFU/g respectively. The lowest moisture content of the tested spices is 4.2 (in candlenut) and 12.8% (in cinnamon) (Figure 1). Fungal contamination of store-dried spices may indicates the ability of storage fungi to grow with low moisture content. It is likely that the level

Table 3. Number of *A. flavus* isolates and their toxigenic strains

	Fungal population										Total
	black pepper	candle nut	cinnamon	cloves	coriander	cardamom	cumin	nutmeg	star anise	white pepper	
A. flavus isolates	8	1	1	1	3	12	1	9	0	14	50
A. flavus toxigenic strain	2	0	0	1	1	10	1	4	0	5	24

https://doi.org/10.17221/18/2019-CJFS

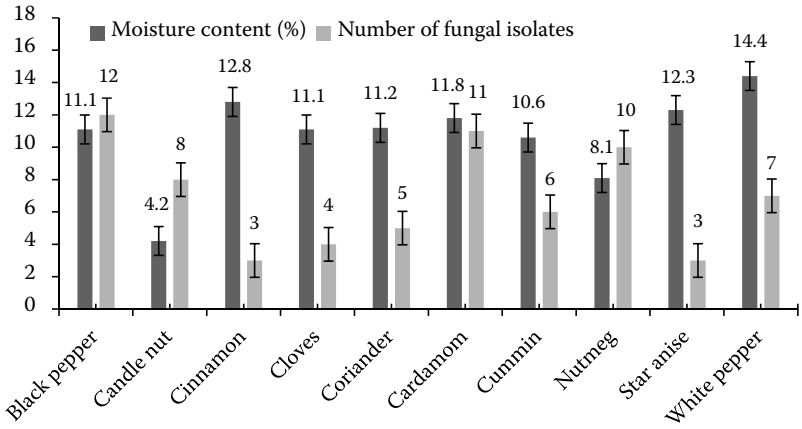

Figure 1. Moisture content and number of fungal genera isolated from spices collected in Medan, North Sumatera, Indonesia

of fungal contamination of store-dried spices was also determined by the presence of chemical compounds on the spices. Dried extract of star anise (*Illicium verum*) inhibits *A. niger*, *A. flavus*, *A. parasiticus*, and *Fusarium moniliforme* (Aly *et al.* 2016). Cinnamon oil inhibits mycelial growth from *R. stolonifer* and *A. niger* (Li *et al.* 2014).

A total of 50 *A. flavus* strains of were isolated from the ten spices studied and all of the strains produced L (large) sclerotia and biseriate conidial heads. The largest number of *A. flavus* isolates was found on white pepper, followed by cardamom, nutmeg kernels, black pepper and coriander with 14, 12, 9, 8, and 3 strains respectively (Table 3).

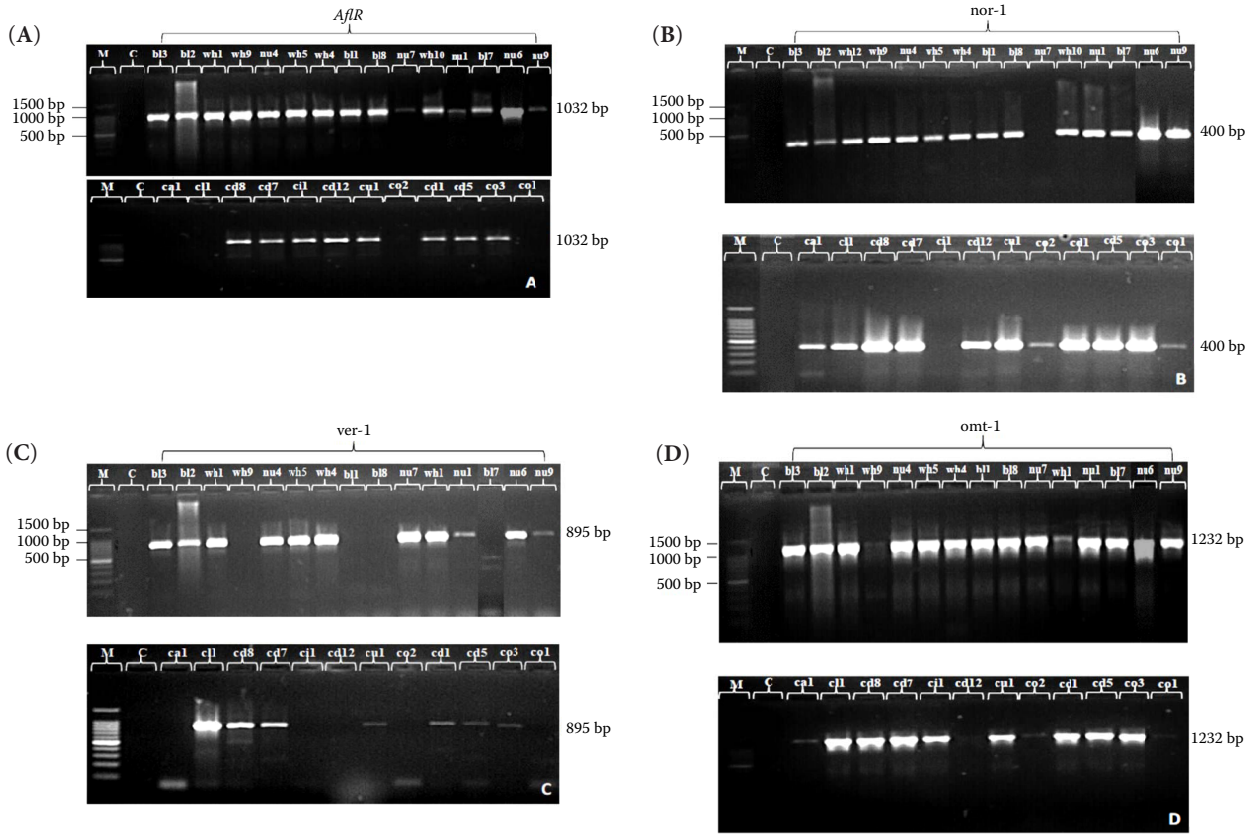

Figure 2. Electrophoresis quadruplex PCR analysis of regulatory gene: *aflR* (**A**) and stuctural genes *nor*-1 (**B**), *ver*-1 (**C**), and *omt*-1 (**D**) extracted from 27 represented toxigenic and non-toxigenic *Aspergillus flavus*

bl – black pepper; wh – white pepper; nu – nutmeg; ca – candle nut; cl – cloves; cd – cardamom; ci – cinnamon; cu – cummin; co – coriander; M – marker ladder (100 bp); C – template control

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Table 4. Toxigenicity of *A. flavus* strains isolated from store-dried spices using molecular-based and cultural-based methods

Store-dried spices	*Aspergillus flavus* strain	Regulatory and structural genes				Yellow pigment in CAM	Toxigenicity
		aflR	*nor*-1	*ver*-1	*omt*-1		
Black pepper	bl1	+	+	–	+	negative	non-toxigen
	bl2	+	+	+	+	positive	toxigen
	bl3	+	+	+	+	positive	toxigen
	bl7	+	+	–	+	negative	non-toxigen
	bl8	+	+	–	+	negative	non-toxigen
Nutmeg	nu1	+	+	+	+	positive	toxigen
	nu4	+	+	+	+	positive	toxigen
	nu6	+	+	+	+	positive	toxigen
	nu7	+	–	+	+	negative	non-toxigen
	nu9	+	+	+	+	positive	toxigen
White pepper	wh4	+	+	+	+	positive	toxigen
	wh5	+	+	+	+	positive	toxigen
	wh9	+	+	–	+	negative	non-toxigen
	wh10	+	+	+	+	positive	toxigen
	wh12	+	+	+	+	positive	toxigen
Cardamom	ca1	–	+	–	+	negative	non-toxigen
	cd1	+	+	+	+	positive	toxigen
	cd5	+	+	+	+	positive	toxigen
	cd7	+	+	+	+	positive	toxigen
	cd8	+	+	+	+	positive	toxigen
	cd12	+	+	–	–	negative	non-toxigen
Cloves	ci1	+	–	–	+	negative	non-toxigen
	cl1	–	+	+	+	negative	non-toxigen
Coriander	co1	–	+	–	+	negative	non-toxigen
	co2	–	+	–	+	negative	non-toxigen
	co3	+	+	+	+	positive	toxigen
Cumin	cu1	+	+	+	+	positive	toxigen

'+' genes were amplified; '–' no amplification, CAM – coconut agar medium

Among the 50 *A. flavus* strains isolated, 48% strains were toxigenic (aflatoxin producers). The number of toxigenic strains varied on each spice. Comparison between toxigenic and non-toxigenic *A. flavus* strains in cardamom was the highest (10 of 12) followed by nutmeg, white pepper, and black pepper. Cloves, cumin, cinnamon and candlenut were slightly contaminated and no *A. flavus* was found on star anise. Further studies (*e.g.* Mohammadpour *et al.* 2012; Aly *et al.* 2016) shows that the natural product present in of the spices might reduce the fungal population).

Gel electrophoresis of quadruplex PCR using specific primers and DNA extracted from the represented toxigenic and non-toxigenic *A. flavus* strains showed varying patterns (Figure 2).

The presence of regulatory and structural genes determines the toxigenicity of the strains. Bands of regulatory (*aflR*) and structural genes (*nor*-1, *ver*-1, *omt*-1) can be seen at 1032, 400, 895, and 1232 bp respectively. The gene of the aflatoxin biosynthetic pathway are found in a cluster Yu *et al.* (2004). Toxigenic strains were indicated by the quadruplex pattern of the four genes as found in black pepper (bl2, bl3), nurmeg (nu1, nu4, nu6, and nu9), white pepper (wh4, wh5, wh10, and wh12), cardamom (cd1, cd5, cd7, and cd8), coriander (co3), and cumin (cu1). The non-toxigenic strains lack bands representing one or more steps in the pathway.

Comparison of the toxigenic and non-toxigenic *A. flavus* strains by molecular based detection and culture-based showing similar results (Table 4).

https://doi.org/10.17221/18/2019-CJFS

The presence of yellow pigment to detect toxigenic *A. flavus* has been previously described by IN and DIANESE (1976) and DAVIS *et al.* (1987). Quadruplex PCR to differentiate toxigenicity *A. flavus* by amplification of regulatory and structural genes in aflatoxin pathway on spices is rapid and applicable.

CONCLUSIONS

Different fungal populations occurred on each kinds of store-dried spices, and *Aspergillus flavus* and *A. chevalieri* were the most prevalent population. More toxigenic *A. flavus* strains were present than non-toxigenic ones. Good handling practices are required to reduce fungal population and prevent fungal contamination and mycotoxin production on stored spices. Future research should investigate the possible anti-fungal components of spices that supported low levels of fungal contamination.

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