Review Paper

Aspergillus niger as the Source of Ochratoxin A of ~~Contamineed~~Contaminated *Pyrus communis* in Taif Market.

Abstract

Fruits are one of the most important agricultural products that supply the body with vitamins and essential minerals elements, but it is attacked by fungi during the period of growth, harvesting and storage. *A. niger* is one of the species that grows on the fruit during the period of storage, and secretes mycotoxins especially ochratoxin A. This study was concerned with the isolation and definition of different strains of *A. niger* from 20 samples of pear collected from Taif markets and the ability of these strains to ~~form~~ produce ochratoxin A. The results showed that 19 samples of pear were contaminated with different strains of *A. niger*. The study showed the ability of this strains to produce ochratoxin A. From 27 isolates of *A. niger* used to test the ability of production ochratoxin A, 10 strains only produced ochratoxin A. The range of OTA in all strains were 0.18 to 9.5 ppb. Representative 27 strains of ochratoxigenic and non ochratoxigenic black aspergilli isolates were subjected for detection of ochratoxin biosynthesis genes, by using two sets of primer for two genes involved in ochratoxin biosynthetic pathway. Bands of the fragments of PKS15C-MeT and PKS15KS genes visualized at 928 and 776 bp, respectively.

Keywords:- black aspergilla, ochratoxin A, *Pyrus communis*, DNA Isolation, PKS15C-MeT and PKS15KS genes

Introduction

Fruits are commercially and nutritionally vital food. Fruits play a vital role in human nutrition by supplied with the required growth factors as vitamins and essential minerals, fats, and oil within the right proportion to take care of growth and development on humans, daily diet maintaining a decent and traditional health[1]. Because of environmental condition, pests, inadequate downfall and fungi attack, fruits and vegetable have serious challenges to their existence[1]. Over the

years, fungal caused several of disease like rot diseases that provoke severe losses of agricultural and horticultural crops each year [2,3]. One of the most vital limiting factors that impact the economic value of fruits is the comparatively short shelf-life period caused by pathogens. About 20-25% of the harvested fruits are deteriorated by pathogens throughout post-harvest handling even in advanced countries [4,5].

Fungi are treated as an important post-harvest losses agent of many different fruits, depended on variety, season and production area amid alternative factors [6,7]. Many of crop diseases are caused by fungi as the most crucial and common pathogens. Fungi colonized many of fruits and vegetables during storage and transportation[8]. Rotted fungi are considered biological agents that have ability to produce a wide range of enzyme, which able these fungi to colonize the fruits. Mould growth depends on several factors such pH, water activity (aw), temperature, atmosphere, time, etc [9].

~~*Aspergillus* A.~~ *niger* is a fungus and one the most widespread species of the genus *Aspergillus.* It causes a disease called black mold on fruits and vegetables similar to grapes, onions and peanuts and is a common contamination of food. *A. niger* is common in soil and many of environments. *A. niger* produce many of mycotoxins such as ochratoxin A [10,11], fumonisins B2, B4 and B6 [12,13,14], as well as numerous other compounds with poorly investigated activities[15,16], such as nephrotoxic, nephrogenic which are immunological in nature. In addition, this fungus is also causative agent for many rot diseases in plants[17]. Black *Aspergilli* (*Aspergillus* section Nigri) are useful in food mycology, medical mycology and biotechnology, often occurring in indoor environments[18,19]. several species of fungi cause food spoilage, however are also utilized in the fermentation industry to provide varied enzymes and organic acids [20].

Isolates of *A.niger* have the ability to produce OTA, then, many concerns have arisen not only for their biotechnological safety but also for their food safety risk, due to their common presence in numerous commodities[21,22,23, 24]. OTA have properties of a potent nephrotoxin and has teratogenic, immunosuppressive and carcinogenic properties[25]. OTA entry to humans and animals is caused by cereals

and cereal based food and feed which considered the main contributors, since OTA is stable under traditional food processing operation conditions and it is carried-over from raw materials to processed products[26]. At recent days, mycotoxin issues has widened, there are many reports showed the ability of *A.niger* to produce fumonisin B2 (FB2) along with OTA[26,27,28]. The International Agency for Research on Cancer classified OTA as a possible carcinogen to humans (group 2B) [25]. Many varieties of food product within the markets are reportable to be contaminated with OTA. These include tree nuts, peanuts, figs, melon seed, pumpkin seed, sesame seed, sunflower seed, lotus seed, corn seed, red pepper, white pepper, mixed spices, rice, corn, mixed cereals, chilies, and coconut [29].

This study aimed for the isolation and definition of different strains of *A.niger* from 20 samples of pear collected from Taif markets and the ability of these strains to form ochratoxin A.

Materials & Methods

Collection of samples:

Twenty samples showing rot symptoms of *Pyrus communis* (Pear) were collected from different markets and vendors in Taif city during October-December 2015 to isolate black aspergilli.

Isolation of black aspergilla

Isolation was performed by serial dilution technique[30], 10 g from *Pyrus communis* samples at the margin of diseased/ healthy tissue were removed and soaked in 100 ml sterilized distilled water that have been put in the shaking incubator for 30 min. Thereafter, 1ml aliquots from serial dilution were inoculated onto three plates containing malt extract agar medium (MEA) and then incubated at 27º C for 5-7 days and the developing fungi were counted and identified. At the end of the incubation period, colonies black aspergilli was counted and were conducted following calculations for account of isolates:

Determination of OTA ability of black aspergilli species isolates:

Ochratoxin-producing ability of the isolates was performed by cultivating black aspergilli in czapek yeast autolysate agar medium (CYA) (g/L; sucrose 30.00, sodium nitrate 2.00, magnesium glycerophosphate 0.50, potassium sulfate 0.35, potassium chloride 0.50, ferrous sulfate 0.01, agar- agar 15.00) supplemented with (5.0 g / L) yeast extract [31] for 5 days at 27° C.

OTA was extracted by grinding the moldy agar (20 g) in blender for 1 min with methanol (100 ml) containing 0.5% NaCl. The mixture was then filtered through a fluted filter paper (24 cm), and the filtrate was diluted (1:4) with 1x 0.1% Tween PBS (Phosphate Buffered Saline) and refiltered through a glass-fiber filter paper. Two milliliters of the glass-fiber filtrate were placed on OchraTest columns (VICAM, Watertown, MA, USA) and allowed to elute at 1-2 drops/sec. The columns were washed two times with 10 ml of 1x 0.1% Tween PBS and 10 ml of Phosphate Buffered Saline (PBS), respectively. Then, OTA was eluted from the column with 1.5 ml OchraTest™ Eluting Solution and OTA concentration was read in a recalibrated VICAMSeries-4 fluorometer after 60 seconds.

Extraction of genomic DNA:

Mycelial cultures were harvested from potato dextrose broth (PDB) grown for 20 to 24 h in 10-ml tubes (3 ml of culture) or at 30° C (225 rpm) by filtering them through Whatman paper (Fisher Scientific, Inc., Pittsburgh, Pa.), washed according to the manufacturer's instructions, and then blotted dry.

DNA extraction was performed with an Epicentre kit but with a modification of the manufacturer's protocol. Approximately 200 mg of washed mycelia was added to a 1.7-ml micro centrifuge tube. The step involving grinding in liquid nitrogen was omitted; instead, 450 µl of yeast cell lysis solution and 1 µl of a 50-µg/ml concentration of proteinase K were added to the tubes. The tubes were vortexed for 10 s, incubated in a 65° C heating block for 1 h, and then chilled on ice for 5 min. Next, 225 µl of protein precipitation reagent was added, and the tubes were vortexed for 5 s. The suspensions were then centrifuged at 20,800 × *g* for 10 min to pellet cellular debris. The supernatant (~500 µl) was transferred to a new tube, spun again to remove any residual cellular material, and then transferred to a new tube. An equal volume of isopropanol was added, and

the tubes were gently inverted several times to precipitate the DNA, which was then pelleted by centrifugation at 20,800g for 10 min. Pellets were washed with 70% ice-cold ethanol, centrifuged, and then vacuum dried. DNA was resuspended in 50 to 100 µl of Tris-EDTA and then treated with 2 µl of a 5-mg/ml concentration of RNase A at 65° C for 1 h[32]. Finally, the DNA quantity and quality were checked by electrophoresis on a 0.8% agarose gel, revealed with ethidium bromide and visualized by UV trans-illumination.

Molecular detection of OTA biosynthetic genes in ochratoxigenic species of black aspergelli:

Two published primer sets were used for the specific detection of two OTA genes.

The first one, denoted PKS15C-MeT (5´GCTTTCATGGACTGGATG and 5´CATTCGTTGATCCCATCG). Reactions were incubated for 2 min at 95°C , followed by 35 cycles of 45s at 94° C, 50s at 62° C and 1 min at 72° C. Amplification cycles finished with 5 min incubation at 72 °C. Expected Results: Amplicon ~SIZE 998 bp only on positive strains.

The second pair, named PKS15KS (5´CAATGCCGTCCAACCGTATG and 5´CCTTCGCCTCGCCCGTAG). Reactions were incubated for 4 min at 94° C, followed by 35 cycles of 45 s at 94° C, 50 s at 60° C and 1 min at 72° C. Amplification cycles finished with 5 min incubation at 72° C. Expected Results: Amplicon ~SIZE 776 bp only on positive strains.

Results

Three species belonging to black Aspergelli were isolated and identified from *Pyrus communis* fruit on MEA medium at 27˚ C (Table, 1and 2).

The total counts of fungi from *Pyrus communis* fluctuated between 0-27 isolates with the highest count being estimated in samples number 14 (27 isolates), while the lowest number of isolates were recovered from samples number 3, 6, 7, and 8 (1 isolate), whereas sample number 4 not contaminated by this species (Table, 2). According to the average total counts (ATC) of all black Aspergilli collected from 20 *Pyrus communis* fruit samples, *A. niger* was the most

common species, which recovered from 70% of the samples, matching 73.4% of total black ~~a~~Aspergilli. In the individual sample the count of this species ranged from 3-23 colonies and the highest count was observed in sample no. 14 (Tables, 1 & 2).

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Quantitative determination of OTA:

All black aspergilli species collected from the investigated samples represented with single isolate from each sample of *Pyrus communis* fruits collectively were tested for OTA potentials. It was detected at varying degrees and estimated by part per billion.

Table (3) showed the results of OTA production, where only two well know ochratoxigenic species were detected (*A. niger* and *A. tubingensis*).

Among isolates of black aspergilli, the ranges of OTA in all strains were 0.18-9.5 ppb. *A. niger* (SNM7 strain) showed the highest level of OTA (9.5 ppb) and *A. niger* (SNM22 strain) showed the lowest level of OTA (0.18 ppb). The production level of OTA from *A. niger* (SNM15 strain), (SNM19 strain), (SNM20 strain) and (SNM25 strain) were 2.4, 2.5, 1.2, and 0.95 ppb, respectively. ~~A~~and the production level of OTA from *A. tubingensis* (SNM13 strain), (SNM16 strain), (SNM17 strain) and (SNM26 strain) were 0.84, 1.2, 0.3 and 0.65 ppb, respectively.

Whereas, OTA disappeared in all *A. awamori* isolates (SNM3 strain), (SNM8 strain), (SNM10 strain) and (SNM18 strain). Also, OTA disappeared from 12 isolates (SNM 1, 2, 4, 5, 9, 11, 12, 14, 21, 23, 24 and 27) of *A. niger* and one isolate (SNM6) of *A. tubingensis*.

Detection of some of OTA biosynthesis genes:

Representative 27 strains of ochratoxigenic and non ochratoxigenic black aspergilli isolates were subjected for detection of ochratoxin biosynthesis genes.

Polymerase chain reaction (PCR) was applied using two sets of primer for two genes involved in ochratoxin biosynthetic pathway. Bands of the fragments of PKS15C-MeT and PKS15KS genes visualized at 998 and 776 bp, respectively (fig. 1).

Table (4) explained the total ochratoxin and ochratoxigenic genes (PKS15C-MeT and PKS15KS) detected in 27 strains of ochratoxigenic black aspergilli isolates collected from *Pyrus communis* samples. From those 27 strains, *A. niger* (SNM7, and 19), and *A. tubingensis* (SNM16, and 26) contained the two OTA biosynthesis genes. But, *A. niger* (SNM20, and 22) contained only PKS15C-MeT gene, while *A. niger* (SNM15, and 25) and *A. tubingensis* (SNM17) contained only PKS15KS gene. On the other hand, 17 non ochratoxigenic strains showed no bands, which means that, there is deletion in targeted genes in this isolates. All *A. awamori* strains (SNM3, 8, 10, and 18), *A. niger* strains (SNM1, 2, 4, 5, 9, 11, 12, 14, 21, 23, 24, and 27), and *A. tubingensis* strain (SNM6) showed no bands.

Discussion

Three species belonging to black Aspergelli were isolated and identified from *Pyrus communis* fruit on MEA medium at 27˚ C. *A.niger* was the most common species, which recovered from 70% of the samples, matching 73.4% of total black aspergilli. In the individual sample the count of this species ranged from 1-23 colonies and the highest count was observed in sample no. 14. *A. niger* var. *niger* and *Aspergillus niger* var. *awamori* were isolated in highest frequency from black dried vine fruits on DRBC and DG18 media. Where OTA was found in 74% of the dried vine fruits samples. *A. carbonarius* occupied the first place in the production of OTA, were detected (82·6%). Followed by *Aspergillus* section Nigri so sixty two strains (28 %) have the ability to produce OTA[33]. The pomegranate trees are not affected by any serious disease however the fruit are often damaged by heart rot caused by different species fungi and bacteria or after invasion of the insect. Twenty-six samples of splitting pomegranate fruits from different garden close to Cairo, Egypt were examined, and they showed that they contain a reproductive structure of genus *A. niger* which can reach the guts of the fruit throughout the period of growth until harvest the mature fruits[34].

Among isolates of black Aaspergilli, the ranges of OTA in all strains were 0.18-9.5 ppb. *A. niger* (SNM7 strain) showed the highest level of OTA (9.5 ppb) and *A. niger* (SNM22 strain) showed the lowest level of OTA (0.18 ppb).

Whereas, OTA disappeared in all *A. awamori* isolates (SNM3 strain), (SNM8 strain), (SNM10 strain) and (SNM18 strain). Also, OTA disappeared from 12 isolates (SNM 1, 2, 4, 5, 9, 11, 12, 14, 21, 23, 24 and 27) of *A. niger* and one isolate (SNM6) of *A. tubingensis*.

Fungi producing OTA in Portuguese wine grapes, a survey was conducted in 11 vineyards, from winemaking regions every with different climatic conditions. They isolated 370 strains of *Aspergillus* and 301 strains of *Penicillium* from 1650 samples of barriers, the study showed 14% of the aspergilli were OTA-producing strains. None of the penicillia were OTA-producing strains. The black aspergilli were predominant (90%). 97 % of black aspergilla were *Aspergillus carbonarius* and 3% of the *Aspergillus niger* collected in this study were OTA producers [35].

many species of fungi were isolated from five grape varieties grown in Spain. The most fungal genera isolated were *Alternaria, Cladosporium*, and *Aspergillus*. The study showed that 82% *Aspergillus* sp. section Nigri were OTA-producing strains, was assessed using yeast extract-sucrose broth supplemented with 5% bee pollen. Cultures of 205 isolates from this section appeared that 74.2% of *Aspergillus carbonarius* and 14.3% of *Aspergillus tubingensis* isolates produced OTA ranging from 1.2 to 3,530 mg/ml and from 46.4 to 111.5 mg/ml, respectively. No *Aspergillus niger* isolate had the ability to produce this toxin under the conditions assayed [36].

Onion bulbs will become contaminated with different species of fungi during the storage period, *Aspergillus* section Nigri was the most important causal agents. standard morphological methods was not enough to identify black Aspergilli, because this group of *Aspergillus* contain many species that cannot be reliably identified by standard morphological methods. Therefore, it's necessary to identify the fungus inflicting this problem within the onion specifically, particularly since some species of section Nigri are well-known as ochratoxin and fumonisin producers. Sixty fungal isolates belonging to10 fungal genera were isolated from 40 onion samples collected from the Taif region in Saudi Arabia from 40 samples of onion, Black aspergilli were isolated from 37 onion

samples. Using primer pairs (awaspec and Cmd6) designed based on partial calmodulin gene sequence data, 37 isolates were identified as *A. welwitschiae* (*A. awamori*).The ochratoxin A and fumonisin B2 contents of the onion samples were examined. No ochratoxins were detected within the collected samples, whereas fumonisin B2 was detected in 37.5 % of the onion samples. Eighteen of thirty seven isolates of genus *Aspergillus welwitschiae* were recognized as potential producers for fumonisin B2. Multiplex polymerase chain reactions designed to detect biosynthetic genes of fumonisins confirmed these results[30].

the total ochratoxin and ochratoxigenic genes (PKS15C-MeT and PKS15KS) detected in 27 strains of ochratoxigenic black aspergilli isolates collected from *Pyrus communis* samples. From those 27 strains, *A. niger* (SNM7, and 24), and *A. tubingensis* (SNM16, and 26) contained the two OTA biosynthesis genes. But, *A. niger* (SNM20, and 22) contained only PKS15C-MeT gene, while *A. niger* (SNM15, and 25) and *A. tubingensis* (SNM17) contained only PKS15KS gene. On the other hand, 17 non ochratoxigenic strains showed no bands, which means that the there is deletion in targeted genes in this isolates. All *A. awamori* strains (SNM3, 8, 10, and 18), *A. niger* strains (SNM1, 2, 4, 5, 9, 11, 12, 14, 21, 23, 24, and 27), and *A. tubingensis* strain (SNM6) showed no bands. According to the results of [37] study, the aflatoxigenic species of *Aspergillus* has been shown to vary in their aflatoxin potentials with the substrate and environmental factors. Whereas, the presence of four tested genes is not sufficient marker for differentiation between aflatoxigenic and non aflatoxigenic isolates.

OTA-nonproducing isolates of *A.niger* and *A.welwitschiae* (*A.awamori*) *species* lacked the ochratoxin A biosynthetic gene (OTA) cluster, analysis of genome sequence data revealed a single pattern of OTA gene deletion in the two species. Phylogenetic analysis suggest that the simplest explanation for this is that OTA cluster deletion occurred in a common ancestor of *A. A. niger* and *A. welwitschiae*, and subsequently both the intact and deleted cluster were retained as alternate alleles during divergence of the ancestor into descendent species. When comparing their results with previous studies indicated that a minority of isolates of both species produce OTA. also, suggested that the relative abundance of each

species and frequency of OTA-producing isolates can vary with crop and/or geographic origin[38].

Conclusion

In this study, black Aspergilli was isolated from 20 samples of *Pyrus communis*. Most of the samples showed to be contaminated with black Aspergilli. The common black Aspergilli is *A. niger*. In detection of the ability of these fungi to produce ochratoxin A, some of them have the ability to form ochratoxin A, the production of these toxins is linked to the presence of one or more genes.

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Table (1): Counts (as colonies in every sample) of *Aspergillus* section Nigri recovered from 20 *Pyrus communis* fruit on MEA medium at 27º C.

Species	Samples																				Total
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
A. awamori	2	0	0	0	2	0	1	0	0	0	0	0	0	0	0	1	1	0	4	0	11
A. niger	10	10	1	0	0	1	0	1	8	0	1	6	13	23	6	11	0	7	0	4	102
A. tubingensis	0	0	0	0	0	0	0	0	3	2	1	5	4	4	3	2	2	0	0	0	26
Gross total count	12	10	1	0	2	1	1	1	11	2	2	11	17	27	9	14	3	7	4	4	139

Table (2): Average total counts (ATC, calculated per g fresh fruit in all samples), percentage counts (%C, calculated per *Aspergillus* section Nigri), percentage frequency (%F, calculated per 20 samples), number of cases of isolation (NCI, out of 20 samples) and occurrence remarks (OR) of various fungal species collected from *Pyrus communis* fruit samples on MEA medium at 27º C.

Species	ATC	C%	NCI	OR	F%
A. awamori	36.7	7.9	6	M	30
A. niger	340	73.4	14	H	70
A. tubingensis	86.7	18.7	9	M	45
Total count	463.4	100			

Occurrence remarks: OR (out of 20 samples), H= high occurrence from 10-20 cases, M= moderate occurrence from 5-9 cases, L= low occurrence from 2-4 cases and R= rare occurrence 1 case.

Table (3): Total Ochratoxin A (PPB) produced by different black aspergilli species isolated from different *Pyrus communis* samples in CYA medium at 27˚ C for 7 days.

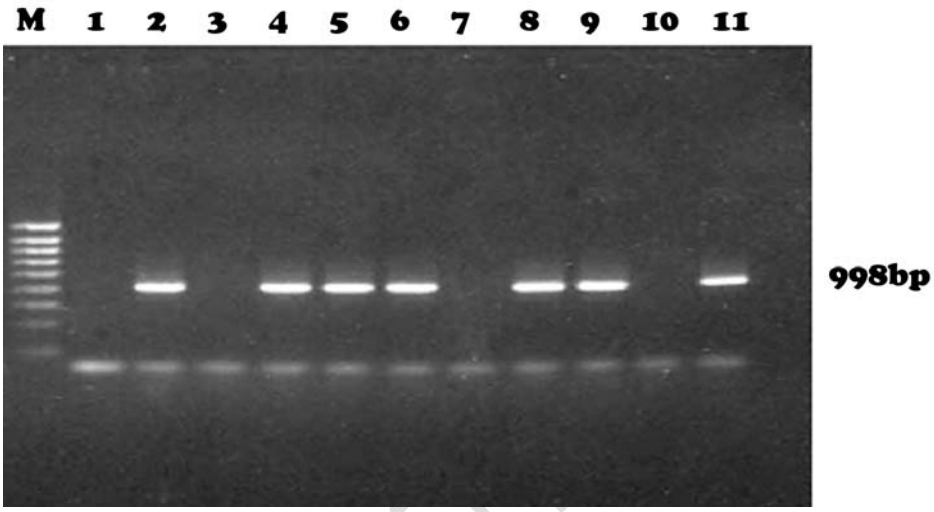
No.	Strain code	Species	OTA level (PPB)
1	SNM3	*A. awamori*	Nd
2	SNM8	*A. awamori*	Nd
3	SNM10	*A. awamori*	Nd
4	SNM18	*A. awamori*	Nd
5	SNM1	*A. niger*	Nd
6	SNM2	*A. niger*	Nd
7	SNM4	*A. niger*	Nd
8	SNM5	*A. niger*	Nd
9	SNM7	*A. niger*	9.5
10	SNM9	*A. niger*	Nd
11	SNM11	*A. niger*	Nd
12	SNM12	*A. niger*	Nd
13	SNM14	*A. niger*	Nd
14	SNM15	*A. niger*	2.4
15	SNM19	*A. niger*	2.5
16	SNM20	*A. niger*	1.2
17	SNM21	*A. niger*	Nd
18	SNM22	*A. niger*	0.18
19	SNM23	*A. niger*	Nd
20	SNM24	*A. niger*	Nd
21	SNM25	*A. niger*	0.95
22	SNM27	*A. niger*	Nd
23	SNM6	*A. tubingensis*	Nd
24	SNM13	*A. tubingensis*	0.84
25	SNM16	*A. tubingensis*	1.2
26	SNM17	*A. tubingensis*	0.3
27	SNM26	*A. tubingensis*	0.65

Nd: Not detected with the limit of detection

Table (4): Total ochratoxin A and ochratoxigenic genes (PKS15C-MeT and PKS15KS) detected in 27 strains of black aspergilli isolates collected from pear samples.

No.	Strain code	Total OTA genes	PKS15C-MeT	PKS15KS
1	SNM1	-	-	-
2	SNM2	-	-	-
3	SNM3	-	-	-
4	SNM4	-	-	-
5	SNM5	-	-	-
6	SNM6	-	-	-
7	SNM7	+	+	+
8	SNM8	-	-	-
9	SNM9	-	-	-
10	SNM10	-	-	-
11	SNM11	-	-	-
12	SNM12	-	-	-
13	SNM13	+	+	+
14	SNM14	-	-	-
15	SNM15	+	-	+
16	SNM16	+	+	+
17	SNM17	+	-	+
18	SNM18	-	-	-
19	SNM19	+	+	+
20	SNM20	+	+	-
21	SNM21	-	-	-
22	SNM22	+	+	-
23	SNM23	-	-	-
24	SNM24	-	-	-
25	SNM25	+	-	+
26	SNM26	+	+	+
27	SNM27	-	-	-

+ Presence
- Absence



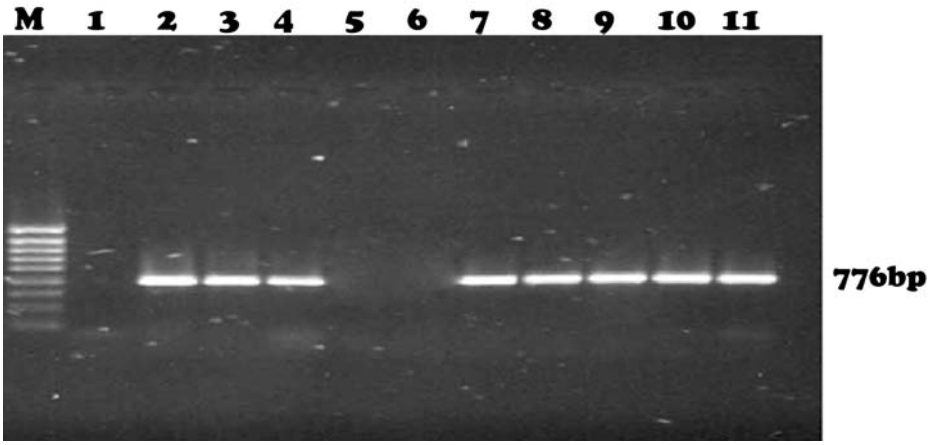


Fig. (1): Ochratoxin biosynthesis genes amplifications. (A). PKS15C-MeT gene and (B). PKS15KS gene. M, DNA marker; Lane 1, negative control; Lanes 2-7, *A. niger* (SNM 7, 15, 19, 20, 22, and 25); Lanes 8-11, *A. tubingensis* (SNM 13, 16, 17, and 26).