Original Research Article

Molecular Screening of Fungal Isolates of Palm Oil from South Eastern Nigeria ~~Palm Oil~~ for Aflatoxin and Ochratoxin biosynthetic genes using Multiplex Polymerase Chain Reaction (mPCR).

Abstract.

In Nigeria and many other developing countries of the world, the incidence of mycotoxin-contamination of foods and food products has attracted attention and stirred a lot of concern for food safety. This work aims at detection of aflatoxigenic and ochratoxigenic synthetic genes from fungal isolates of palm oil as a veritable means for the evaluation of foods for possible mycotoxin contamination. In this study, fungal isolates from palm oil samples collected from the five states of South-east geopolitical zone in Nigeria were screened for aflatoxin and ochratoxin biosynthetic genes using Multiplex Polymerase Chain Reaction (mPCR). The assay relied on three sets of primers that amplify aflatoxgenic *Aspergillus*, ochratoxigenic *Aspergillus* and *Penicillium* species under optimized PCR conditions. Optimum multiplex PCR assay was standardized for simultaneous detection of toxigenic *Aspergillus* and ochratoxin producing *Penicillium* species targeting *AflR*, *AflS* and polyketide synthase *(pks)* genes involved in aflatoxin and ochratoxin metabolic pathways respectively. *AflR* primer pair gave specific amplification for aflatoxigenic *A. flavus* but did not give amplification for *A. niger* and *P. chrysogenum*. While *Afl*S and *pks* gave amplification for only aflatoxigenic and ochratoxigenic *A. niger* and *P. chrysogenum*. In the evaluation and monitoring of mycotoxin-producing fungi during the processing of food and feed commodities, Multiplex PCR approach could be a veritable tool to supplement the conventional analytical techniques.

Keywords: Palm oil, Aflatoxin, Ochratoxin, Multiplex PCR, *AflR*, *AflS*, *pks*.

1. Introduction.

The palm oil ~~palm~~, an economic tree and as the most important source of edible oil ranks among the top most oil producing crops in Sub-saharan Africa. One of the major oils and fats traded in the different continents of the world today is produced from palm fruits [1].

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A mature and ripe palm fruit is reddish in colour and made up of an oily, fleshy outer layer (Pericarp), with a single seed (palm kernel) which is as well rich in oil. Oil is extracted from both the pulp of the fruit and the kernel [2,3].

Palm oil can support the growth of microorganisms, especially lipophilic microbes. The activities of some of these organisms are known lead to deterioration in biochemical quality of the oil [4].

Palm oil may harbour different genera or species of fungi that may be toxigenic or atoxigenic. Filamentous fungi, *Aspergillus* and *Penicillum* are capable of producing mycotoxins such as aflatoxins and ochratoxins in foods which are toxic and carcinogenic when consumed by humans and animals. These fungi might also be present without the presence of toxins and that may imply that the organisms present may not have the genes that are responsible for the production of mycotoxins of concern. A fungus may produce several mycotoxins and a mycotoxin may as well be produced by different fungi [5].

Comment [U1]: Citation needed here

Aflatoxins are toxic, carcinogenic compounds produced by *Aspergillus flavus, Aspergillus parasiticus, and Aspergillus nomius*. Contamination of various commodities by aflatoxins can occur as a result of crop infection by one of these fungi. Animal and human health concerns about aflatoxin-tainted commodities have resulted in stringent regulations worldwide on aflatoxin content; these regulations on aflatoxin contamination have a significant economic impact [6].

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The economic impact of Ochratoxin (OTA) on food commodities is very significant in that OTA producing fungi have been implicated ~~-~~to be a contaminant in a wide variety of foodstuffs [7]. *Aspergillus* and *Penicillium* species are the main producers of ochratoxins. Ochratoxin A (OTA) is a mycotoxin that is receiving increasing attention worldwide because of its severe nephrotoxicity [8].

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The harmful effects of mycotoxins to humans and animals have necessitated the urgent need to develop highly specific and rapid approaches for the detection of mycotoxins in food and food products. To achieve this, molecular techniques ha~~s~~ve been introduced as powerful tools for detecting and identifying fungi. When genes involved in the biosynthetic pathway are known, they represent a valuable target for the specific detection of toxigenic fungi [9]. Multiplex PCR is used for simultaneous detection and amplification of multiple genes [7]. The aim of this work ~~is~~ was to determine the incidence of aflatoxigenic, and ochratoxigenic species of *Aspergillus* and

Penicillum isolated from palm oil sampled from three different markets in five states of South-East geo-political zone in Nigeria.

2.0 Materials and methods.

2.1 Fungal species, media and growth conditions.

The fungal species investigated were isolated in previous characterization studies of palm oil collected from three (3) open markets in five (5) different states of the South-east geo-political zone in Nigeria which include; Abia, Imo, Anambra, Enugu and Ebonyi States. A total of 165 fungal species were isolated and included *Aspergillus flavus*, *A. niger* and *Penicillum chrysogenum.* They were maintained in potato dextrose agar (PDA) slant at 4^{O}C and were sub-cultured periodically.

2.2 Fungal DNA Extraction.

Template DNA was extracted according to methods previously described by Lathe *et al.*, [10] from 3-5 day pure fungal cultures. The fungal mycelia (*A. flavus*, *A. niger*, *A. niger* p and *P. chrysogenum*) were picked ~~with~~ using a wire loop into 1.5ml centrifuge tubes containing 1000µL of phosphate buffered saline (duplicate samples), they were centrifuged at 4000rpm for 2mins. Hipes lysis buffer (400µL) and proteinase K (10µL) were added and vortexed for 15sec. They were then placed in the heat block (Barnstead/Thermolyne Type 17600 Dri-bath, Made in USA.), covered ~~with the heat block cover~~ and incubated at 65^{O}C for 1hr. The tubes were then removed and vortexed after every 20mins to expose the DNA in the mycelia. The DNA was separated using 400µL of phenol - chloroform (1:1), vortexed for 10sec and centrifuged at 14,000rpm for 10min. The supernatants were extracted with a micropipette into clean 1.5ml tubes, avoiding the white interphase. They were further separated with chloroform (400µL), vortexed for 10sec and centrifuged at 14,000rpm for 5min. The supernatants were thereafter ~~extracted with~~collected using a pipette and transferred into another set of 1.5ml tubes, avoiding the white interphase. The polysaccharides and the proteins in the supernatants (the DNA in solution) were precipitated using 1000µL of absolute ethanol (100) and 40µL of 3M sodium acetate and mixed by inverting the tubes. They were incubated at -20^{O}C overnight and centrifuged in a cold centrifuge (4^{O}C) for 10mins. The supernatant (ethanol layers) was discarded; the DNA which is the bottom layer was washed with 400µL of ethanol (70%), and centrifuged in the cold centrifuge for 5min. The

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supernatant (ethanol layers) was discarded and they were centrifuged again to remove all traces of ethanol ~~with~~ using a micropipette. The tubes (containing the DNA pellets) were kept open and left to air-dry for 20mins. DNA samples were re-suspended in 100µL of phosphate buffer.

2.3 Primer design.

Primers were designed using the NCBI/BLAST designing tool on the NCBI site and Sequencer 5.4.6. [11]. The *AflR* primer set was obtained from regions reported for *AflR* genes (Ehrlich *et al.*, 2003). The primers were designed from *AflR and AflS* which are involved in the regulation of aflatoxin biosynthesis and *pks* which is also responsible for ochratoxin biosynthesis. *Afl*R, *AflS* and *pks* primers were used for the specific detection of aflatoxigen (*AflR* and *AflS*) and ochratoxigen (*pks*) producing *Aspergillus* and *Penicillium* species, respectively. Primer sequences are listed in the table1. The *Afl*R, *AflS* and *pks* were obtained from conserved regions reported for *Afl*R, *AflS* and *pks* genes. The primer pairs were imported from BIO NEER, USA. The whole aim was to combine the three primer sets into a single PCR reaction.

2.3.1 Optimization of the primers

The primer sets (Stock: *AflR*, *AflS* and *pks*) in the tubes were centrifuged at 14,000 rpm for 1min., and reconstituted to 100pmoles/L as follows: 105µL of DH_2O was added to *AflR* for., 105µL to *AflR* rev., 104µL to *AflS* for., 108µL to *AflS* rev., 105µL to *pks* for., and 104L to *pks* rev. They were centrifuged for 10sec for even distribution. The primers were diluted to 10pmolar/L as follows: 2µL of each constituted primer (*AflR* for, *AflR* rev, *AflS* for, *AflS* rev, *Pks* for, and *Pks* rev) from the six primer tubes was dispensed into one centrifuge tube (12µL of the combined diluted primers). It was centrifuged for 10 sec for even distribution. Five (5µL) microliters of the combined diluted primers and 45µL of water were dispensed into another centrifuge tube (10pmoles/L i.e. the working primer).

Multiplex PCR Assay

2.3.2 DNA quantification

DNA quantification was done Spectrophotometrically (GeneQuant. *pro*, Biochrom Limited Cambridge, CB4 OFJ, England). The machine was blanked with water (water was poured in the cuvette and measured in the machine). Three (3µL) microliters each of the extracted DNA was transferred into the cuvette and the OD was taken in the spectrophotometer at 260nm to

determine their DNA concentration and ratio in order to determine the quantity and ratio of DNA each organism possess. The cuvette was rinsed properly with water and dried with cotton wool after each use during measurement.

2.3.3 Dilution of template DNA

Four (4µL) microliters of each isolated template DNA (*A. flavus*, *A. niger* and *P. chrysogenum*) were pipetted into another centrifuge tube and 26µL of DH_20 were added. The OD readings were taken on the spectrophotometer at 260nM.

2.3.4 Standardization of mPCR Assay.

To determine the best PCR condition to carry out the analysis, 2µL of the diluted DNA (*A. flavus*) was dispensed into the two Multiplex PCR (mPCR) tubes containing the Multiplex PCR premix (1U of Taq DNA polymerase, dNTP mix (250µM each), reaction buffer, 2.0mM $MgCl_2$, stabilizer and tracking dye ++), 3µL of the diluted primers and 15µL of water. The mPCR tubes were centrifuged with the micro centrifuge after adding a drop of mineral oil into each tube. One tube was kept in the PCR with the following conditions: initial denaturation at 94^OC for 4min, followed by 35 cycles at 94^OC for 1min, primer annealing at 55^OC for 1min and extension at 72^OC for 30 sec. and final extension at 72^OC for 8min in a DNA thermal cycler (PTC 100™, Programmable Thermal Controller; MJ RESEARCH INC.). While the second tube was kept in the PCR with the following conditions: initial denaturation of 94^OC for 4mins, followed by 30 cycles at 94^OC for 1min, primer annealing at 58^OC for 1min. and extension at 72^OC for 30 sec, with incubation at 72^OC for 8min in a DNA thermal cycler (PTC 100, Programmable Thermal Controller; MJ RESEARCH INC.).

2.3.5 Multiplex PCR Conditions

The multiplex PCR tubes contain the Multiplex PCR premix, 15µL of DH_2O, 3µL of each diluted primer specific to the targeted genes (*AflR*, *AflS* and *pks*) and 2µL of each template DNA. The negative control tube contains the Multiplex PCR premix, 17µL of DH_2O and 3µL of diluted primer. They were centrifuged with micro centrifuge (LX-100 Micro Centrifuge Model) after adding a drop of mineral oil into the tubes to prevent the PCR product from evaporating. The tubes were placed in the PCR machine. The PCR cycling conditions were carried out with an initial denaturation of 95^OC for 4mins, followed by 30 cycles at 95^OC for 1min, primer annealing

at 58^{O}C for 1min and extension at 72^{O}C for 30 sec. and final extension at 72^{O}C for 8min in a DNA thermal cycler (PTC 100, Programmable Thermal Controller; MJ RESEARCH INC.).

2.3.6 Gel electrophoresis

The PCR products were analyzed by gel electrophoresis using 1% agarose gel in TAE (40mM Tris base, 40mM acetic acid and 1.0mM EDTA at pH 8.0). The agarose gel (1g of agarose in 100ml 1 X TAE and Ethidium Bromide (5L)) was cut and placed on the gel cast. They were kept in the gel electrophoresis box (Model B1A, OWL SEPARATION SYSTEMS, INC. PORTMOUTH NH USA.) and the box was filled with buffer (1 X TAE) which covered the agarose gel. The 100bp DNA Ladder (8µL) was loaded in the first well on the agarose gel; 8µL of the DNA samples were loaded on the other wells including the negative control. The tank was covered and left to run for 28min at 95volts. Ethidium bromide-stained gels were visualized under UV light and documented in a gel-doc system (GEL DOCUMENTATION SYSTEM Made by BIO-RAD LABORATORIES SEGRATE (MILAN) ITALY) with CCD camera attached to it and connected to a desktop computer.

3.0 Results.

The primer-pairs designed, their sequences, optimal annealing temperature used and the amplicon size (PCR product length) are as shown on Table 1. Primers and annealing temperature (58^{O}C) were standardized to ensure a uniform amplification of the genes targeted for mPCR assay. The mPCR assay conditions were standardized, and the result showed that the best conditions were: initial denaturation of 94^{O}C for 4min, followed by 30 cycles at 94^{O}C for 1min, primer annealing at 58^{O}C for 1min and extension at 72^{O}C for 30 sec with incubation at 72^{O}C for 8min which produced clear bands while the other condition produced dull/faint bands.

Shown in Table 2 is the DNA concentration and DNA ratio of the entire isolated DNA template before and after dilution. The DNA concentration was reduced because high concentration exerts negative effect, resulting in non-amplification.

The aflatoxigenic genes detected by the mPCR assay are shown in Table 3. The result indicates the presence of aflatoxigenic genes in isolates from palm oil samples from Ekeonunwa Owerri Imo state (6C), and Akpugo market (19A and 19B), Eke Emene (20A), Ngwo market (21 A, B and C) in Enugu State.

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185 Table 1: Details of the target genes, primer sequences, annealing temperatures and product

186 length in base pairs (bp) for PCR analysis.

ORGANISM	TARGET GENES	PRIMER CODE	PRIMER SEQUENCE (5′ 3′)	OPTIMAL ANNEALING TEMP. (^{O}C)	PCR PRODUCT LENGTH (bp)
A. flavus	*AflR*	*AflR* for	CCACGATGGTTGACCATATC	58	1036
		AflR rev	TCGGACACGGTGGCGGGACT		
	AflS	*AflS* for	GTGGAGGATACGCTCACTCG		356
		AflS rev	GCGACTCGCATGGTGATTTC		
	Pks	*Pks* for	CGGCCAGGACATTGATACCT		308
		Pks rev	CCGCTATACGGTAGCAGTTG		
A. niger	*AflR*	*AflR* for	CCACGATGGTTGACCATATC	58	1036
		AflR rev	TCGGACACGGTGGCGGGACT		
	AflS	*AflS* for	GTGGAGGATACGCTCACTCG		356
		AflS rev	GCGACTCGCATGGTGATTTC		
	Pks	*Pks* for	CGGCCAGGACATTGATACCT		308
		Pks rev	CCGCTATACGGTAGCAGTTG		
A. niger p	*AflR*	*AflR* for	CCACGATGGTTGACCATATC	58	1036
		AflR rev	TCGGACACGGTGGCGGGACT		
	AflS	*AflS* for	GTGGAGGATACGCTCACTCG		356
		AflS rev	GCGACTCGCATGGTGATTTC		
	Pks	*Pks* for	CGGCCAGGACATTGATACCT		308
		Pks rev	CCGCTATACGGTAGCAGTTG		
P. chrysogenum	*AflR*	*AflR* for	CCACGATGGTTGACCATATC	58	1036
		AflR rev	TCGGACACGGTGGCGGGACT		
	AflS	*AflS* for	GTGGAGGATACGCTCACTCG		356
		AflS rev	GCGACTCGCATGGTGATTTC		
	Pks	*Pks* for	CGGCCAGGACATTGATACCT		308
		Pks rev	CCGCTATACGGTAGCAGTTG		

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Table 2. DNA Quantification Readings Obtained Spectrophotometrically.

S/N	MICROORGANISM	DNA CONCENTRATION (g/ml)		DNA RATIO	
		BEFORE DILUTION	AFTER DILUTION	BEFORE DILUTION	AFTER DILUTION
1	*A. flavus*	88.4	3.6	1.436	1.007
2	*A. niger*	127.9	5.2	1.401	0.982
3	*A. niger* P	116.6	4.7	1.390	0.975
4	*P. chrysogenum*	84.0	3.4	1.348	0.945

The multiplex PCR amplification of *AflR*, *AflS* and *pks* of *A. flavus*, *A. niger* and *P. chrysogenum* showed positive correlation for aflatoxin production where a complete pattern with two bands was observed on agarose gel (Fig.1) and negative correlation for ochratoxin production where no bands were observed. The aflatoxigenic *Aspergillus* (*A. flavus*) gave positive amplification with both *AflR* and *AflS* primer pairs (Fig.1, Lane1) while other fungal isolates did not show amplification with these primer pairs.

Non-ochratoxigenic *Aspergillus* (*A. niger*) and *Penicillum* (*P. chrysogenum*) investigated did not show amplification with *pks* primer pair indicating the absence of ochratoxin producing machinery (Fig.1, Lanes 2, 3 and 4). The result of the mPCR amplification of *AflR* (1036bp) genes from all the isolated *A. flavus* in the different samples is shown in Fig. 2.

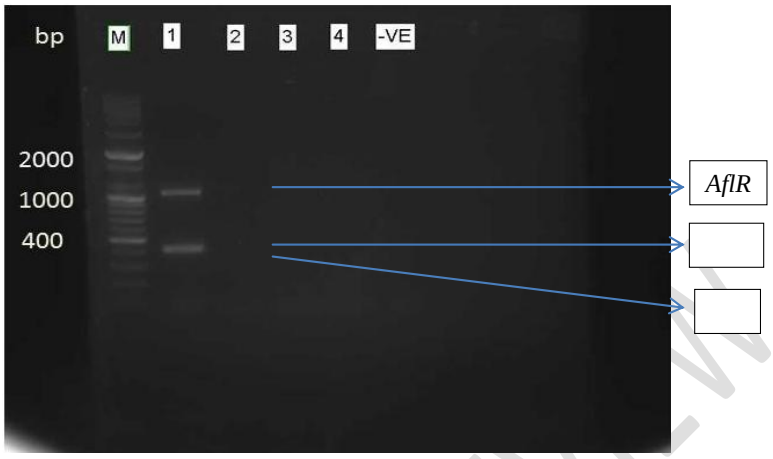

Figure 1: mPCR amplification of *AflR* (1036bp), *AflS* (356bp) and *pks* (308bp) genes from different fungal species. Lanes (M) Marker 100bp, (1) *A. flavus*, (2) *A. niger*, (3) *A. niger* P, (4) *P. chrysogenum*, (-ve) negative control.

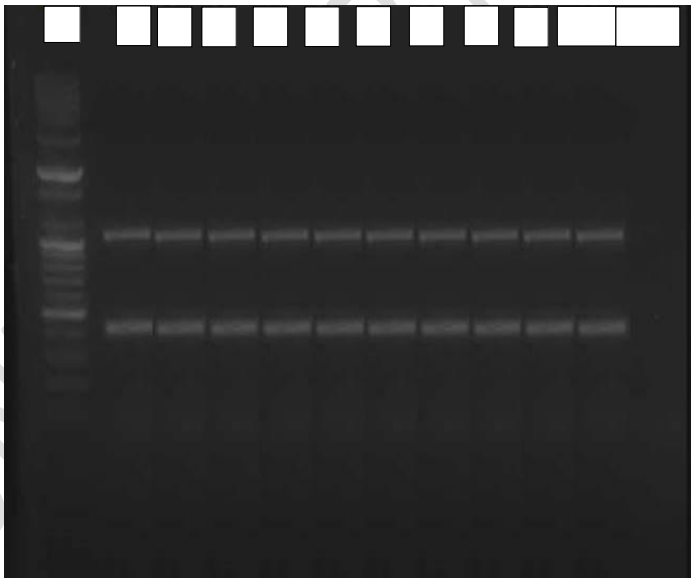

Figure 2: mPCR amplification of *AflR* (1036bp) from isolated *A. flavus*. Lanes (M) Marker 100bp, (1-10) *A. flavus* isolates and (11) negative control.

214 **Table 3. Presence of Aflatoxin and Ochratoxin genes, Aflatoxin and Ochratoxin production**
215 **in collected samples.**

FUNGAL ISOLATES	MYCOTOXIN PRODUCTION	Gene presence detected by Multiplex PCR		
		AflR	*AflS*	*Pks*
1A	ND	-	-	-
1B	ND	-	-	-
1C	ND	-	-	-
2A	ND	-	-	-
2B	ND	-	-	-
2C	ND	-	-	-
3A	ND	-	-	-
3B	ND	-	-	-
3C	ND	-	-	-
5A	ND	-	-	-
5B	ND	-	-	-
5C	ND	-	-	-
6A	ND	-	-	-
6B	ND	-	-	-
6C	AF	+	+	-
7A	ND	-	-	-
7B	ND	-	-	-
7C	ND	-	-	-
9A	ND	-	-	-
9B	ND	-	-	-
9C	ND	-	-	-
10A	ND	-	-	-
10B	ND	-	-	-
10C	ND	-	-	-
11A	ND	-	-	-
11B	ND	-	-	-
11C	ND	-	-	-
15A	ND	-	-	-
15B	ND	-	-	-
15C	ND	-	-	-
16A	ND	-	-	-
16B	ND	-	-	-
16C	ND	-	-	-

17A	ND	-	-	-
17B	ND	-	-	-
17C	ND	-	-	-
19A	AF	+	+	-
19B	AF	+	+	-
19C	ND	-	-	-
20A	AF	+	+	-
20B	ND	-	-	-
20C	ND	-	-	-
21A	AF	+	+	-
21B	AF	+	+	-
21C	AF	+	+	-

Key: - = Non amplification of concerned gene. + = Amplification of concerned gene

Note: 6C – Ekeonunwa Owerri Imo state; 19A and 19B – Akpugo market; 20A – Eke Emene; 21 A, B and C- Ngwo market all in Enugu State.

3.1 Discussion.

In this study, multiplex PCR assay was used to facilitate the screening for aflatoxigenic and ochratoxigenic genes in fungal species isolated from palm oil samples commercially sold in selected markets in South-Eastern Nigeria. ~~Three sets of primer were assembled for the molecular detection of aflatoxin and ochratoxin synthetic genes in the fungal isolates~~. The assay relied on three sets of primers that amplify aflatoxgenic *Aspergillus*, ochratoxigenic *Aspergillus* and *Penicillium* species genes under optimized PCR conditions.

The primer concentrations for the multiplex PCR were optimized for the respective target genes before standardization of the multiplex PCR assay. The multiplex PCR was then optimized with an annealing temperature of 58°C (Table 1). The concentrations of all the oligonucleotide primers targeting various genes were diluted after reconstitution to provide equal intensities of all the amplified products (Table 2). The standardized mPCR revealed the presence of two (2) bands representing *AflR* and *AflS* after agarose gel electrophoresis (Fig. 1) on *A. flavus* and no bands on the other organisms. The target genes, primer sequences, annealing temperatures and product length in base pairs (bp) are depicted in Table 1.

The genes targeted were *AflR* (1036 bp size) encoding transcriptional regulation factor for aflatoxin producing fungi, *AflS (*356 bp size) encoding transcriptional regulation factor for aflatoxin producing fungi and *pks* gene (308bp size encoding polyketide synthase) for ochratoxigenic *Aspergillus* species and *Penicillium* species detection. The results (Fig.1 and 2) indicate that *A. flavus* isolates are aflatoxigenic because they contained the *AflR* and *AflS* genes which is responsible for aflatoxin biosynthesis, but do not contain the ochratoxigenic gene *pk~~-~~s*. ~~w~~ *A. niger* and *P. chrysogenum* are both non-aflatoxigenic and non-ochratoxigenic because they do not contain the aflatoxigenic genes (*AflR* and *AflS*) and the ochratoxigenic gene (*pks*).

The results are in consonance with the report of Sudharsan *et al.*, [13]. The authors assessed the occurrence of toxigenic fungi and mycotoxin contamination in stored wheat grains by using advanced molecular and analytical techniques (optimized mPCR). Aflatoxin B1, fumonisins, and deoxynivalenol were the most common toxins found in these samples. A multiplex polymerase chain reaction (PCR) strategy was developed for rapid screening and identification of mycotoxigenic fungi. The optimized multiplex PCR assay was highly specific in the detection of fungal species containing species-specific and mycotoxin metabolic pathway genes [13]. Similar result was obtained by Latha *et al.*, [9], in which multiplex PCR assay was used for the detection of aflatoxigenic and non- aflatoxigenic *Aspergilli.* All the aflatoxigenic Aspergilli gave positive amplification with *OMT* (o- methyl transferase) and *AFLR* primer pairs. Non-aflatoxigenic *Aspergilli* screened did not show any amplification with *OMT* and *OFLR* primer pairs, indicating the absence of aflatoxin producing machinery.

Many isolates of *A. niger* and *P. chrysogenum* strains may be non-aflatoxigenic because of mutation in one or more genes belonging to the biosynthetic gene cluster [14]. Different types of mutation might be responsible for inactivating the ochratoxigenic pathway genes in *A. niger* and *P. chrysogenum*. A biological approach involving competition of other fungal species led to a reduction in the aflatoxin-producing ability of *Aspergillus flavus* [15].

Though the conventional methods of screening and differentiating aflatoxin- producing *Aspergilli* from non- aflatoxigenic *Aspergillus* in foods and food materials could differentiate aflatoxin- producing strains from the non- aflatoxin producers. However, the methods show low sensitivity, sometimes fails to detect aflatoxin production because of instability of aflatoxin -

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producing strain growing on culture media [9]. Rapid molecular technique such as multiplex PCR is a valuable tool for screening foods and feeds for mycotoxin- producing fungi [16].

3.2 Conclusion

Multiplex PCR assay is a sensitive, rapid and reliable tool that could simultaneously and specifically detect and differentiate mycotoxin-producing fungal strains from non-producers with high sensitivity. In the evaluation and monitoring of mycotoxin-producing fungi during the processing of food and feed commodities, Multiplex PCR approach could be a veritable tool to supplement the conventional analytical techniques for determining food safety.

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