



Molecular Detection of Aflatoxin Producing Strains of *Aspergillus Flavus* from Peanut (*Arachis Hypogaea*)

Adeela Hussain¹, Awais Afzal¹, Muhammad Irfan^{1*}, Kauser Abdulla Malik¹

¹Department of Biological Sciences, Forman Christian College University (A Chartered University), Ferozpur Road, Lahore, Pakistan

ARTICLE INFO

Article history:

Received 08 April 2014

Accepted 27 January 2015

Available online, ISSN: 2148-127X

Keywords:

Aflatoxin

Aspergillus flavus

Peanuts

Contamination

Molecular approach

ABSTRACT

Aflatoxins are the potential carcinogens produced as secondary metabolites by *Aspergillus flavus*. They have the ability to contaminate large number of food which ultimately affect the human population. Malt extract agar was selected for the growth of control stains of fungus. The aim of the study was to develop a reliable and quick method for the detection of aflatoxin producing strains in peanuts by using molecular approaches. Total 80 samples of infected peanuts were collected from four different cities of Punjab and checked for their aflatoxin contamination. For aflatoxin detection, three target genes *nor1*, *ver1* and *aflR* were selected which was involved in the aflatoxin biosynthesis. In all examined cases, 24 out of 80 (30%) samples successfully amplified all three genes indicating aflatoxigenic activity. Discrimination between aflatoxigenic and non-aflatoxigenic strains were also determined on the basis of amplification of these three target DNA fragments. In this study, it was also demonstrated that only specific strains were able to produce the aflatoxin contamination in peanuts.

* Corresponding Author:

E-mail: muhammadirfan@fccollege.edu.pk

Introduction

Mycotoxins are the cluster of chemically toxic secondary metabolites of fungi and have immense potential to infect a large range of food and animal feed. Approximately 25-50% of the crops were reported to be contaminated by mycotoxins worldwide after harvesting (Konietzny and Greiner, 2003). Among these mycotoxins, aflatoxins are the most important and recognized group for polluting agricultural commodities. Aflatoxins are the secondary metabolites and potent carcinogens produced by various species of *Aspergillus* especially *Aspergillus flavus* and *Aspergillus parasiticus* (Ehrlich et al., 2003; Yu et al., 2004). Aflatoxins were first identified in *Aspergillus flavus* hence named as "a" from *Aspergillus* and "fla" from *flavus* due to its origin (Bradburn et al., 1994). These were known as potential immunosuppressant's which caused aflatoxicosis by affecting animal and human health after the ingestion of aflatoxins (Williams et al., 2004). Economic losses of corn, cottonseed, peanuts, sorghum, wheat, rice and other commodities were largely caused by contamination due to aflatoxins (Cotty and Jaime-Garcia, 2007). Contamination by aflatoxins commonly occurs in hot and humid climate basically during development of crop or storage of corn, peanuts and cottonseeds (Yu et al., 2004).

Peanuts are regarded as an important agricultural crop. The oil seed crop *Arachis hypogaea* is commonly known as groundnut or peanut belonging to family leguminosae and sub family Papilionacea (Ahmad and Rahim, 2007). According to an estimate in Pakistan, about 84% of total groundnut is cultivated in Punjab province. Peanut and peanut products are reported to be highly susceptible to aflatoxin contamination in a country like Pakistan due to hot prevailing conditions. Among the mycotoxins, aflatoxins are the potential members which mostly contaminate the peanuts (El-Maghraby and El-Maraghy, 1987).

Conventional methods used for the detection of aflatoxins are microbiological identification, high-performance liquid chromatography (HPLC), thin layer chromatography (TLC) or enzyme-linked immunosorbent assay (ELISA) (Shapira et al., 1996). These methods are mostly time consuming and requiring labor although they are considered as reliable for detection of aflatoxins. However, nowadays polymerase chain reaction (PCR) is being used for the rapid finding of aflatoxins as it is easy to employ, rapid and accurate (Sweeney et al., 2000). Hence, a precise, responsive and specific detection system can be devised on the basis of genes accountable for

aflatoxin biosynthesis by PCR technique in grains and foods affected by aflatoxigenic strains (Shapira et al., 1996; Isabel et al., 2012).

In this study, first step was to detect and optimized the genes (*nor1*, *ver1* and *aflR*) involved in the pathway of aflatoxin biosynthesis in different strains of *Aspergillus flavus*. Then peanut samples were collected from four different regions of Punjab to check the aflatoxigenic and non-aflatoxigenic contamination by using molecular approach. The purpose is to develop a quick and reliable detection method to check the aflatoxin production regarding food safety.

Materials and Methods

Fungal Strains

Different strains of *Aspergillus flavus* i.e. 537, 1002, and 1110 were collected from department of Plant Pathology and Mycology, Punjab University, Lahore.

Growth Conditions

Fungal strains were cultured on three different types of the media i.e. Malt extract agar (MEA) (Oxoid, United Kingdom), Coconut agar (CA) and Yeast peptone dextrose agar (YPD) (Sigma Aldrich, USA) for growth of these strains. The plates were streaked and incubated at 28°C for 4 days. The cultures were revived after every 20-25 days.

Fungal DNA Extraction

A loop full of fungal cultures was inoculated into the Malt extract broth and incubated at 28°C on shaking incubator (150rpm) for 3 days. The mycelia were harvested from the broth by filtration using Whatman filter paper #1 and rinsed with 0.85% solution of NaCl two times followed by centrifugation. Small aliquots of mycelia in the eppendorfs were stored at -20°C for further use. An autoclaved pestle and mortar was pre-cooled at -70°C and 2-3g of washed mycelia grounded into fine powder by using liquid nitrogen. Lysis buffer were added at the ratio of 10ml per gram and heated on water bath at 68°C for 45min with occasional mixing. The eppendorf tubes were centrifuged at 13000rpm for 20min at 4°C. The supernatant was transferred into new eppendorf and one volume of cold 4M sodium acetate (pH 5.2) added to precipitate the polysaccharides and proteins. After mixing it properly the solution was incubated at -20°C for 20min following centrifugation at 13000rpm for 15min at 4°C and clean supernatant was transferred to a fresh eppendorf tube. For DNA pellet, one volume of chilled iso-propanol was added into it, mixed gently and incubated at -20°C for 10min and again followed by centrifugation at

13000rpm for 15min at 4°C. The supernatant was discarded and DNA pellet was washed with 1.0ml of cold 70% ethanol and centrifuged at 13000rpm for 10min at 4°C. The pellet was air dried and dissolved in 30-50 µl of TE buffer depending upon the pellet size and stored at -20°C. The quality and quantity of DNA was analyzed by resolving the samples on gel electrophoresis (Rodrigues et al., 2007).

Detection of Aflatoxin Producing Strains by Molecular Methods

For molecular diagnostics of aflatoxin producing strains of *Aspergillus flavus*, polymerase chain reaction (PCR) was carried out for three genes i.e. *nor1*, *ver1*, and *aflR*. These genes and their primer sequences were selected from the already reported data (Rashid et al., 2008). The name of genes, their primer sequences and their product size is shown in Table 1. All PCR reagents were supplied by Fermentas (USA), primers designed from Thermo scientific and all PCR attempts were carried out in PCR Thermal Cycler (Applied Biosystems).

Optimization of PCR

PCR reaction conditions were optimized separately for these three target genes. Reaction mixture was made 25µl on ice-box by adding 2.5µl 10X PCR buffer (+KCl), 1.5mM MgCl₂, 0.1mM dNTPs, 1U *Taq polymerase* (1.0U/µl), 0.4pmol of each forward and reverse primers (20pmol), 1µl (80ng) of template DNA and 17.5µl double distilled water. Thermal cycle conditions were carried as mentioned (Fig 1) except annealing temperature which was variable (58-62°C) for these three genes. PCR amplified products were checked on 1% gel by gel electrophoresis and visualized under Gel documentation system.

Detection of aflatoxin in peanuts

Samples of peanuts were collected from four different regions of Punjab (Lahore, Sahiwal, Bahawalpur and Rahim Yar Khan) for their aflatoxin analysis. First the uninfected peanuts were taken and artificially inoculated with *Aspergillus flavus* strains to check the potential of these strains for aflatoxin production in peanuts. Fungal DNA extraction was done and PCR reactions for three targeted genes were performed by the same method as above. After that, the PCR analysis of infected samples was done in which samples were proceeded directly for DNA extraction of fungal mold. PCR was also performed on the infected samples which were placed for incubation at 28°C for 24hr. PCR amplified products were checked on 2% gel by gel electrophoresis and visualized under Gel documentation system.

Table 1 PCR Primers used for the three genes involved in Aflatoxin Biosynthesis

Gene Name	Primer Name	Sequence 5'-3'	Product size	References
<i>Nor (aflD)</i>	nor1	ACCGCTACGCCGGCACTCTCGGCAC	400bp	Rashid et al., (2008)
	nor2	TTGGCCGCCAGCTTCGACACTCCG		
<i>Ver (aflM)</i>	ver1	GCCGCAGGCCGCGGAGAAAGTGGT	600bp	Rashid et al., (2008)
	ver2	GGGATATACTCCC GCGACACAGCC		
<i>AflR</i>	aflR1	TATCTCCCCCGGGCATCTCCCGG	1000bp	Rashid et al., (2008)
	aflR2	CCGTCAGACAGCCACTGGACACGG		

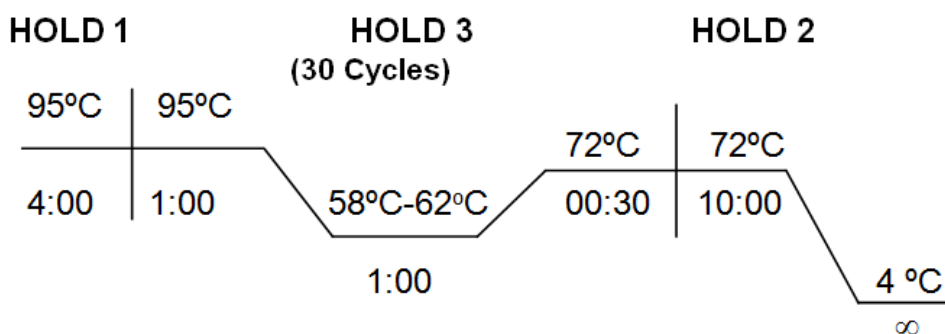


Fig 1 PCR temperature condition for *nor1* (58°C), *ver1* (58°C) and *aflR* (60°C)

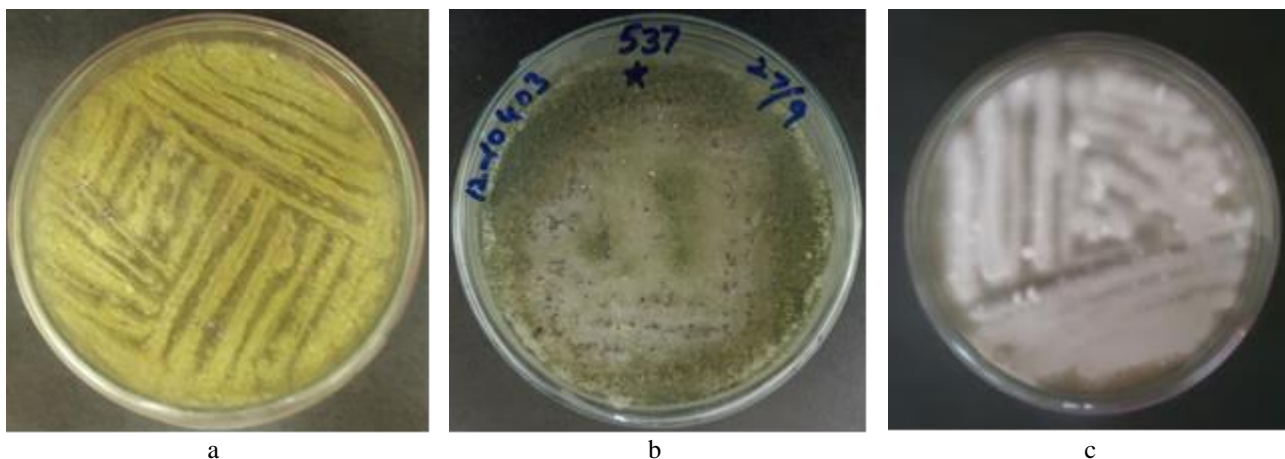


Fig 2 (a) Growth of *A.flavus* on MEA; (b) Growth of *A.flavus* on CA; (c) Growth of *A.flavus* on YPD

Table 2 Frequency of aflatoxin contamination in various cities of Punjab

Sampling city	Sample size	PCR +ve	Percentage (%)
Lahore	20	3/20	15
Sahiwal	20	5/20	25
Bahawalpur	20	7/20	35
Rahim Yar Khan	20	9/20	45
Total	80	24	30



Fig 3 Fluffy mycelia growth of *A. flavus* in ME broth after incubation of 3 days

Results

For strains conservation, they were cultured on three different types of the media (MEA, CA and YPD) and all three showed good growth (Fig 2a, 2b, 2c). For further experiment the fungal cultures were maintained on Malt extract agar (MEA).

For DNA extraction enrichment culture of Malt Extract broth (ME) was used which showed the phenomenal growth of fungal mycelia in the form of spherical fluffy mass (Fig 3). From these fungal masses DNA was extracted successfully. Optimization of the three targeted genes were done by using four different annealing temperatures (56, 58, 60 and 62°C). For PCR diagnostic approach, two structural genes i.e. *nor1* and *ver1* gave the amplification of 400bp at 58°C and 600bp at 58°C and regulatory gene *aflR* gave the amplification of 1000bp at 60°C, respectively (Fig 4a, 4b). All the three strains showed positive PCR results for all three targeted genes.

Peanuts were chosen as sample to diagnose aflatoxigenic molds growth by using molecular approaches. The uninfected peanuts were first artificially inoculated to check whether the peanuts got affected by these three *A. flavus* strains. Some of the peanuts got infected effectively (Fig 5). Only one strain i.e. 537 was able to show aflatoxigenic growth on peanut which was confirmed by PCR and this strain was used as control or reference strain (Fig 6). It also indicated that particular strains of *A. flavus* infect the specific commodities.

Total 80 infected samples of old peanuts from four different cities of Punjab were taken. DNA was extracted successfully from fungal mold which was infecting these samples. Out of 80, 24 (30%) samples showed the aflatoxin contamination by amplifying the three target genes. Some of the results are illustrated in the (Fig 7) and (Table 2).

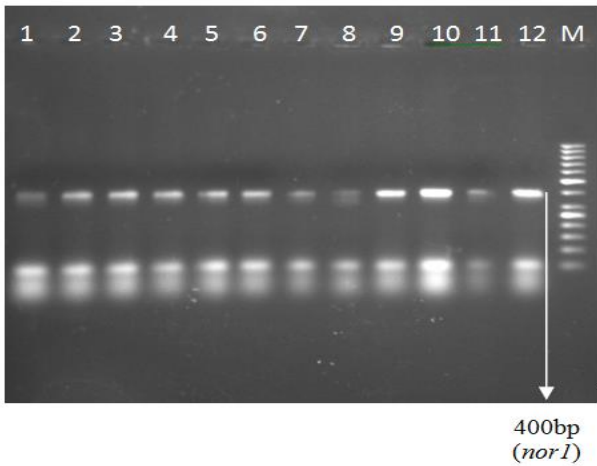


Fig 4 (a) PCR amplified product of *nor1* gene in 537, 1002 and 1110 strains of *A. flavus* at different temperatures.

M: 50bp DNA ladder; **Lane 1-4:** Amplification of *nor1* gene in strain-537 at 56, 58, 60 and 62°C respectively; **Lane 5-8:** Amplification *nor1* gene in strain-1002 at 56, 58, 60 and 62°C respectively; **Lane 9-12:** Amplification *nor1* gene in strain-1110 at 56, 58, 60 and 62°C respectively

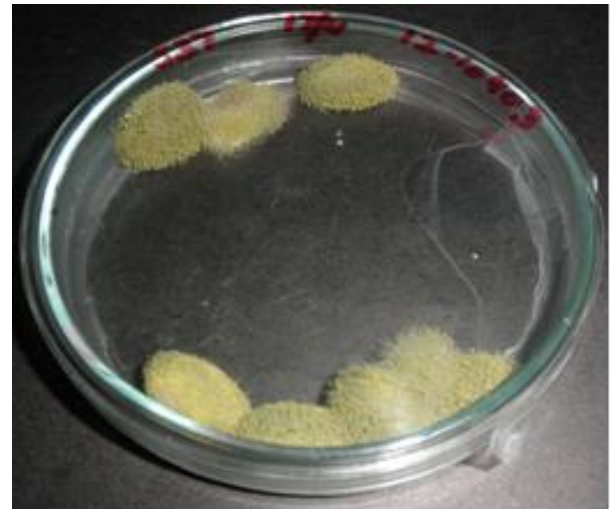


Fig 5 Infection of peanut with aflatoxigenic strain-537

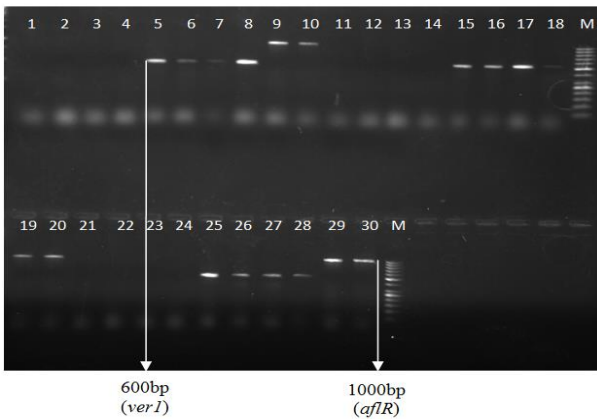


Fig 4 (b) PCR amplified products of *ver* and *aflR* in strains 537, 1002 and 1110 of *A. flavus* at different temperatures.

M: 50bp DNA Ladder; **Lane 1-4:** Negative control at 56, 58, 60 and 62°C for 537; **Lane 5-8:** Amplification of *ver1* gene in strain-537 at 56, 58, 60 and 62°C respectively; **Lane 9-10:** Amplification of *aflR* gene in strain-537 at 58 and 60°C respectively; **Lane 11-14:** Negative control at 56, 58, 60 and 62°C for 1002; **Lane 15-18:** Amplification of *ver1* gene in strain-1002 at 56, 58, 60 and 62°C respectively; **Lane 19-20:** Amplification of *aflR* gene in strain-1002 at 58 and 60°C respectively; **Lane 21-24:** Negative control at 56, 58, 60 and 62°C for 1110; **Lane 25-28:** Amplification of *ver1* gene in strain-1110 at 56, 58, 60 and 62°C respectively; **Lane 29-30:** Amplification of *aflR* gene in strain-1110 at 58 and 60°C respectively

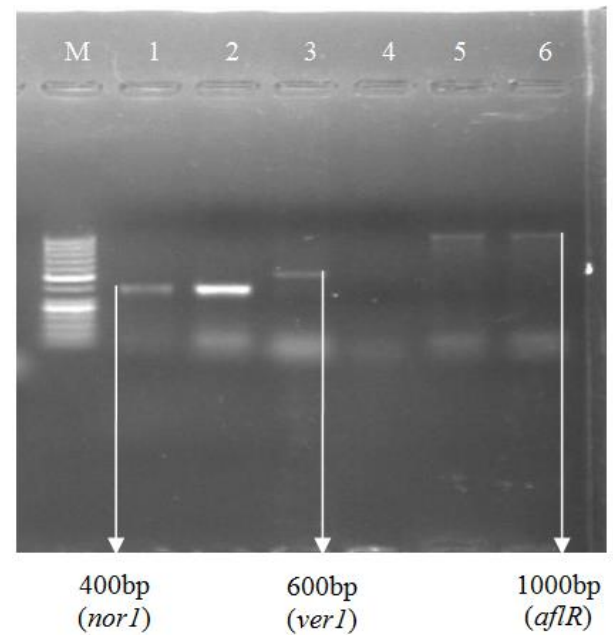


Fig 6 PCR amplification of *nor1*, *ver1* and *aflR* genes in the inoculated peanuts by strain-537

M: 50bp DNA ladder; **Lane 1-2:** Amplification of *nor* gene in artificially inoculated peanuts by strain-537 at 56 and 58°C respectively; **Lane 3:** Amplification of *ver* gene in artificially inoculated peanuts by strain-537 at 56°C; **Lane 4:** Negative control ; **Lane 5-6:** Amplification of *aflR* gene in artificially inoculated peanuts by strain-537 at 58 and 60°C respectively

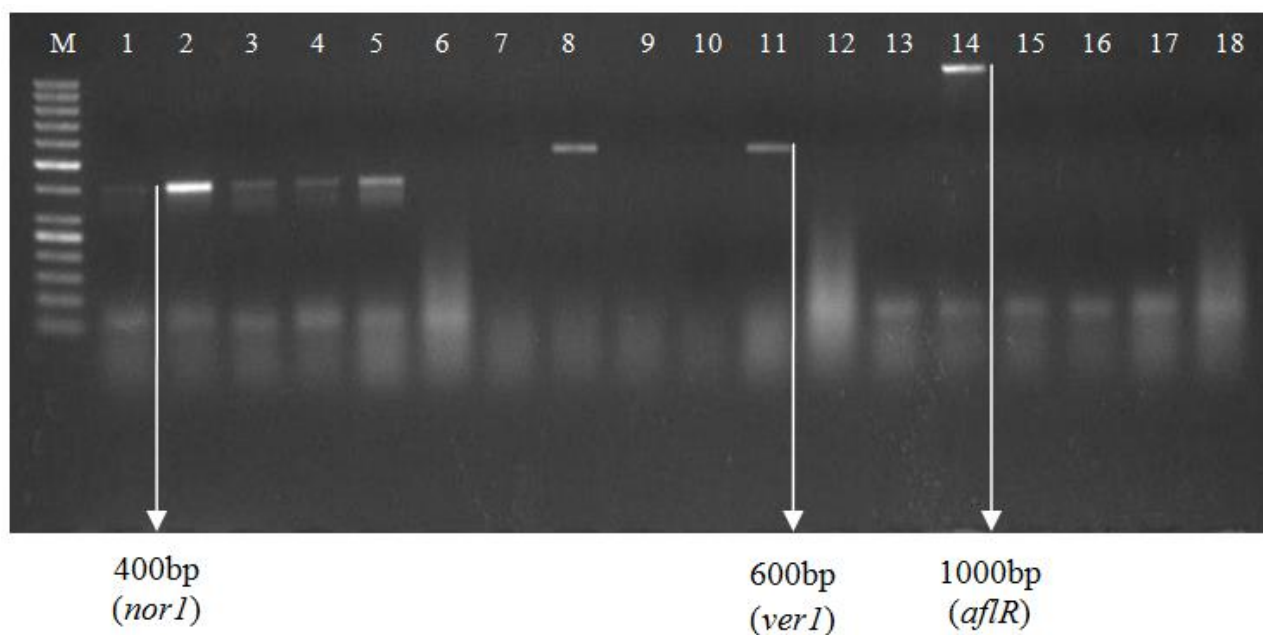


Fig 7 Amplified bands for Sample PCR amplification of *norI*, *verI* and *aflR* at 58°C

M: 50bp DNA ladder; Lane 1-5: PCR amplification of *norI* gene in samples A, B, C, D, E and F; Lane 8 and 11: PCR amplification of *verI* gene in samples B and E; Lane 14: PCR amplification of *aflR* gene in sample B; Lane 6,7,9,10,12,13,15,16,17 and 18: No positive result

Discussion

In the present study, we have developed a rapid, sensitive and specific strategy to be used in food matrix for the detection of *Aspergillus flavus* contaminated with aflatoxins. The approach allows to process a high number of samples and to obtain results in a short time in comparison with conventional methods.

Different types of media were being used for growth and culture preservation but Malt Extract Media (MEM) gave the best possible growth of *A. flavus* strains. (Rodrigues et al., 2007; Paterson, 2007; Bokhari and Magda, 2009; Hadi et al., 2011).

Aspergillus flavus had been the prevalent species among *Aspergillus* section (Horn and Dorner 1999; Trucksessy et al., 2002; Vaamonde et al., 2003; Bankole et al., 2004). This study was conducted because recently *A. flavus* was found to be most commonly isolated from peanuts and was the most common mycotoxigenic component of the mycobiota (Sultan and Magan, 2010). Regardless of this, no attempt was made to detect aflatoxin producing species by using rapid molecular techniques in peanuts. (Bhattacharya and Raha, 2002; González et al., 2008; Nakai et al., 2008; Pildain et al., 2008).

The most common methods used for the detection were microbiological identification, ELISA, chromatography and fluorescence. But these techniques had the main disadvantage of requiring well equipped laboratories, skilled labour, hazardous solvents and more time to complete an assay. For that matter, in this study, we specifically detected *A. flavus* by PCR amplification from contaminated stored peanuts.

To increase the DNA amount and obtaining fresh colonies, enrichment method was used in which fungi

from different sources was incubated in enrichment medium as described by Schadd et al., (1995). Enrichment step was followed by DNA extraction which was now easily extractable for the PCR based detection of aflatoxigenic fungi.

Biosynthetic pathway comprises of many enzymatic steps with aflatoxins as the end product. Despite earlier work, there is yet to be identified a specific marker for aflatoxin production, apart from its measurement (Geisen 1996; Shapira et al., 1996; Farber et al., 1997; Criseo et al., 2001; Chen et al., 2002; Scherm et al., 2005; Degola et al., 2007; Rodrigues et al., 2009).

Several papers have reported use of PCR technology as rapid and sensitive method for detection and diagnosis of aflatoxin production (Hadi et al., 2011) and to detect aflatoxigenic strain from non-aflatoxigenic strain (Rodrigues, 2007; Rashid et al., 2008) in other food commodities apart from peanuts. PCR analysis was able to amplify two structural genes i.e. *norI* and *verI* and regulatory gene *aflR* by using specially designed primers. *aflR* is a positive regulatory gene which is required for transcriptional activation of most of the structural genes (Liu and Chu, 1998; Yu et al., 2004).

Fungal flora of peanuts in Punjab was isolated and tested for its aflatoxin contamination. 10 isolates out of 40 were aflatoxin producers when tested by PCR analysis. In this study it was observed that *norI* gene was being amplified in almost all of them as it was a structural gene required in the initial step. Three primers were carefully selected to be highly specific for these three genes known to be essential for aflatoxin biosynthesis. Each primer pair yielded a single DNA fragment of the expected size of 400, 600 and 1000bp for *norI*, *verI* and *aflR*,

respectively. In this study, all the three strains were able to show the amplification of these three genes. These genes have been tested as molecular markers for aflatoxin production but with variable level of success which can be achieved by numerous attempts of PCR (Shapira et al., 1996; Criseo et al., 2001; Chen et al., 2002; Hinrikson et al., 2005; Rodrigues et al., 2007).

Aflatoxigenic activity from the old peanut samples was tested in which 24 samples out of 80 were gave the positive results for aflatoxin contamination. Non-aflatoxigenic samples presented different patterns by showing no amplification. All the samples having less than three target DNA fragments were considered to be non-aflatoxigenic.

In this study, it is concluded that all the three strains of *Aspergillus flavus* i.e. 537, 1002, and 1110 were able to confirm the aflatoxin production by amplifying the three target genes (*nor1*, *ver1* and *aflR*) as these genes are considered as indicators of aflatoxin production. In the present study, molecular method is proved as rapid and accurate detection system to differentiate the aflatoxigenic and non-aflatoxigenic *A. flavus* strains. It is also demonstrated in this study that particular strain only affects the peanuts and show aflatoxigenicity and 24 out of 80 (30%) infected old peanut samples are proved to show the aflatoxin contamination by amplifying the selected target genes. As Pakistan is an agriculture country and 30% aflatoxin contamination rate in peanuts is commendably high in just four cities hence a diagnostic kit can be prepared for quick detection in fields.

Acknowledgements

The support of Muhammad Hasnain and Nufaid Khan is gratefully acknowledged and highly valued.

References

- Ahmad N, Rahim M. 2007. Evaluation of promising groundnut *Arachis hypogaea* L. varieties for yield and other characters. *J. Agric. Res.*, 45:185-189.
- Bankole SA, Ogunsanwo BM, Mabekoje OO. 2004. Natural occurrence of moulds and aflatoxin B1 in melon seeds from markets in Nigeria. *Food and Chem. Toxicol.*, 42:1309-14.
- Bhattacharya K, Raha S. 2002. Deteriorative changes of maize, groundnut and soybean seeds by fungi in storage. *Mycopathol.*, 155:135-141.
- Bokhari F M, Aly M. 2009. Trials towards reduction of fungal growth and aflatoxin G1 production in Arabic coffee using different additives. *Afr. J. Food Sci.*, 3:068-076.
- Bradburn N, Coker RD, Blunden G. 1994. The etiology of turkey 'x' disease. *Phytochem.*, 35: 817-817.
- Chen RS, Tsay JG, Huang YF, Chiou RYY. 2002. Polymerase chain reaction-mediated characterization of molds belonging to *Aspergillus flavus* group and detection of *Aspergillus parasiticus* in peanut kernels by a multiplex polymerase chain reaction. *J. Food Prot.*, 65: 840-844.
- Cotty PJ, Jaime-Garcia R. 2007. Influences of climate on aflatoxin producing fungi and aflatoxin contamination. *Intl. J. Food Microbiol.*, 119:109-115.
- Criseo G, Bagnara A, Bisignano G. 2001. Differentiation of aflatoxin-producing and non-producing strains of *Aspergillus flavus* group. *Lett. App. Microbiol.*, 33:291-295.
- Degola F, Berni E, Dall'Asta C, Spotti E, Marchelli R, Ferrero I, Restivo FM. 2007. A multiplex RT-PCR approach to detect aflatoxigenic strains of *Aspergillus flavus*. *J. Appl. Microbiol.*, 103: 409-417.
- Ehrlich KC, Montalbano VG, Cotty PJ. 2003. Sequence comparison of *aflR* from different *Aspergillus* species provides evidence for variability in regulation of aflatoxin production. *Fung. Genet. Biol.*, 38: 63-74.
- El-Maghraby OMO, El-Maraghy M. 1987. Mycoflora and mycotoxins of peanuts (*Arachis hypogaea* L.) seeds in Egypt. I-sugar fungi and natural occurrence of mycotoxins. *Mycopathol.*, 98: 165-170.
- Färber P, Geisen R, Holzapfel WH. 1997. Detection of aflatoxinogenic fungi in figs by a PCR reaction. *Intl. J. Food Microbiol.*, 36: 215-220.
- Geisen R. 1996. Multiplex polymerase chain reaction for the detection of potential aflatoxin and sterigmatocystin producing fungi. *Syst. and App. Microbiol.*, 19: 388-392.
- Gonçalves E, Nogueira JHC, Fonseca H, Felício JD, Pino FA, Corrêa B. 2008. Mycobiota and mycotoxins in Brazilian peanut kernels from sowing to harvest. *Intl. J. Food Microbiol.*, 123:184-190.
- Hadi AA, Carter D, Magan N. 2011. Discrimination between aflatoxigenic and non-aflatoxigenic *Aspergillus* section *Flavi* strains from Egyptian peanuts using molecular and analytical techniques. *World Myco. J.*, 4: 69-77.
- Hinrikson HP, Hurst SF, de Aguirre L, Morrison CJ. 2005. Molecular methods for the identification of *Aspergillus* species. *Med. Mycol.*, 43:129-37.
- Horn BW, Dorner JW. 1999. Regional differences in production of aflatoxin b1 and cyclopiazonic acid by soil isolates of *Aspergillus flavus* along transect within the United States. *App. and Environ. Microbiol.*, 65:1444-1449.
- Isabel ML, Alicia R, Andrade MJ, Martín A, Córdoba JJ. 2012. Development of a PCR Protocol to Detect Aflatoxigenic Molds in Food Products. *J. Food Prot.*, 75: 85-94.
- Konietzny U, Greiner R. 2003. The application of PCR in detection of mycotoxigenic fungi in food. *Braz. J. Microbiol.*, 34: 283-300.
- Liu BH, Chu FS. 1998. Regulation of *aflR* and its product associated with aflatoxin biosynthesis. *App. Environ. Microbiol.*, 10: 3718-22.
- Nakai VK, de Oliveira L, Rocha A, Gonçalves E, Fonseca H, Ortega EMM, Corrêa B. 2008. Distribution of fungi and aflatoxins in a stored peanut variety. *Food Chem.*, 106: 285-290.
- Paterson RRM. 2007. Aflatoxins contamination in chilli samples from Pakistan. *Food Ctrl.*, 18: 817-820.
- Pildain BM, Frisvad JC, Vaamonde G, Cabral D, Varga J, Samson RA. 2008. Two novel aflatoxin-producing *Aspergillus* species from Argentinean peanuts. *Intl. J. Syst. and Evol. Microbiol.*, 58: 725-735.
- Rashid M, Khalil S, Ayub N, Ahmed W, Khan AG. 2009. Categorization of *Aspergillus flavus* and *Aspergillus parasiticus* isolates of stored wheat grains into aflatoxinogenics and non-aflatoxinogenics. *Pak. J. Bot.*, 40: 2177-2192.
- Rodrigues P, Venencio A, Kozakiewicz Z, Lima N. 2009. A polyphasic approach to the identification of aflatoxigenic and non-aflatoxigenic strains of *Aspergillus* section *Flavi* isolated from Portuguese almonds. *Intl. J. Food Microbiol.*, 129: 187-193.
- Rodrigues P, Soares C, Kozakiewicz Z, Paterson RRM, Lima N, Venâncio A. 2007. Identification and characterization of *Aspergillus flavus* and aflatoxins. IN: Méndez-Villas A. (Editor) – "Communicating Current Research and Educational Topics and Trends in Applied Microbiology". *Formatex.*, 527-534.

- Schaad NW, Cheong SS, Tamaki S, Hatziloukas E, Panopoulos NJ. 1995. A combined biological and enzymatic amplification (BIOPCR) technique to detect *Pseudomonas syringae* pv. *phaseolicola* in bean extracts. *Phytopath.*, 85: 243-248
- Scherm B, Palomba M, Serra D, Marcello A, Migheli Q. 2005. Detection of transcripts of the aflatoxin genes *aflD*, *aflO* and *aflP* by reverse transcriptase-polymerase chain reaction allows differentiation of aflatoxin-producing and non-producing isolates of *Aspergillus flavus* and *Aspergillus parasiticus*. *Int. J. Food. Microbiol.*, 98: 201-210.
- Shapira R, Paster N, Eyal O, Menasherov M, Mett A, Salomon R. 1996. Detection of aflatoxigenic moulds in grains by PCR. *App. Environ. Microbiol.*, 62:3270-3273.
- Sultan Y, Magan N. 2010. Mycotoxigenic fungi in peanuts from different geographic regions of Egypt. *Mycotoxin Res.*, 26: 133-140.
- Sweeney MJ, Pamies P, Dobson ADW. 2000. The use of reverse transcription-polymerase chain reaction (RT-PCR) for monitoring aflatoxin production in *Aspergillus parasiticus*. *Intl. J. Food Microbiol.*, 56: 97-103.
- Trucksessy MW, Dombrink-Kurtzman MA, Tournasy VH, White KD. 2002. Occurrence of aflatoxins and fumonisins in *Incaparina* from Guatemala. *Food Addit. Contam.*, 7: 671-675.
- Vaamonde G, Patriarca A, Fernández-Pinto V, Comerio R, Degrossi C. 2003. Variability of aflatoxin and cyclopiazonic acid production by *Aspergillus* section *flavi* from different substrates in Argentina. *Intl. J. Food Microbiol.*, 88:79-84.
- Williams JH, Phillips TD, Jolly PE, Stiles JK, Jolly CM, Aggarwal D. 2004. Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. *American J. Clin. Nutr.*, 80: 1106-1122.
- Wyss P, Bonfante P. 1993. Amplification of genomic DNA of arbuscular- mycorrhizal (AM) fungi by PCR using short arbitrary primers. *Mycol. Res.*, 97: 1351-1357.
- Youssef MS, El-Maghraby OMO, Ibrahim YM. 2008. Mycobiota and mycotoxins of Egyptian peanut (*Arachis hypogaea* L.) seeds. *Intl. J. Bot.*, 4: 349-360.
- Yu J, Chang PK, Ehrlich KC, Cary JW, Bhatnagar D, Cleveland TE, Payne GA, Linz JE, Woloshuk CP, Bennett W. 2004. Clustered pathway genes in aflatoxin biosynthesis. *App. Environ. Microbiol.*, 70: 1253-1262.