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RESEARCH ARTICLE

Molecular Identification of Black *Aspergilli* Isolated from Poultry Feeds by Sequencing their ITS-Regions

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ABSTRACT

Black *Aspergilli* are second most important cause of contamination of poultry feed and their by-products with ochratoxin-A after *Aspergillus ochraceous* in Pakistan. A total of 23 black *Aspergilli* were isolated from poultry feeds. Of these black *Aspergilli* samples, 12 (52.17%) were toxigenic (Ochratoxin A). A PCR based molecular method of cloning and sequencing of ITS (internal transcribed spacer) regions of fungal DNA was developed to identify these fungi. A universal pair of primers (ITS1 and ITS4) was used and PCR product (600 bp) for *A. niger* was amplified. These specific universal primers of ITS region were tested for twelve DNA samples of different isolates of *Aspergillus* species isolated from poultry feeds. The sequencing of ITS-region provided a powerful and reliable tool for identification of black *Aspergilli* responsible for the production of ochratoxin-A in poultry feed.

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INTRODUCTION

Contamination of food and agricultural commodities by various types of toxigenic fungi is a serious and widely neglected problem (Bhat *et al*., 2010; Zia *et al*., 2010).The black *Aspergilli* are among the most common fungi causing feed spoilage. Presence of black *Aspergilli* is usually linked to the presence of ochratoxin A. *A. carbonarius*, one of the black fungi, is highlighted as main specie responsible for ochratoxin production (Sage *et al*., 2002; Bau *et al*., 2005). Black *Aspergilli* are one of the most difficult groups concerning identification and classification. Taxonomic study of black *Aspergilli* section is very important in these days and different species concept has been prevailing. Morphological identification of *A. niger* from each other have always been very difficult (Martinez and Culebras, 2007). The internal transcribed spacer regions (ITS) from the 5.8S RNA are non-coding and hyper variable among the different species. These sequences are conserved and are useful for measuring close phylogenetic relationship in fungi (White *et al*., 1990). Because ribosomal regions evolve in concerted way they display a low intra-specific variability, which has been proved useful for identification of *Aspergillus* species (Accensi *et al*., 1999). Therefore, increasing use of molecular approaches in diagnosis has provided tools for answering taxonomic questions that morphological procedure left unsolved (Taylor *et al*., 1999; Parenicova *et al*., 2000). A talented and informative molecular technique for characterization of *Apergillus* and *Penicillium* species is the use of sequence analysis of variable conserved DNA regions like 28S, ITS-1/ITS2, β tubulin, calmodulin and elongation factor (Geiser *et al*., 2000).

In Pakistan, so far, little published information is available about fungal mycobiota in agricultural products particularly those used as ingredients in poultry and animal feeds. Only sporadic reports covering short periods and smaller regions described the presence of some toxigenic fungi or mycotoxins in agricultural products (Saleemullaha *et al*., 2006), poultry, and animal feed stuffs (Hanif *et al*., 2006; Saleemi *et al*., 2010). However, none of the workers has reported application of molecular markers for the identification of closely related species.

In the absence of sufficient basic data, recommendations for control and prevention of contamination of feed ingredients by toxigenic fungi cannot be made. Therefore, the main objective of this study was to explore and apply molecular tools for identification of closely related *Aspergillus* species isolated from poultry feeds.

MATERIALS AND METHODS

Sample Collection, Morphologic Identification: Details of sample collection, fungal isolation and morphologic identification have been published earlier (Saleemi *et al*., 2010). Briefly, 119 samples of poultry feeds were collected from Faisalabad region and inoculated on Potato dextrose, Czapek dox, Czapek yeast autolysate agars for development of fungal colonies. Different fungal species were identified by microscopic examination of slide cultures of fungal colonies.

Genomic DNA extraction: Fungal DNA was extracted by modified method of Cenis (1992). Fungal strains were grown in Vogel's medium (Vogel, 1956) at 28°C for 72 h and mycelia were collected by centrifugation (8000 rpm) at 4°C. For each fungal strain, fresh collected mycelia were ground gently, in liquid nitrogen with the help of sterilized mortar and pestle. Five grams of ground mycelia were re-suspended in 10 ml of extraction buffer (200 mM Tris-HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) in 50 ml sterilized tube. Sodium acetate (5 mL, 3M) was added in the same tube and stored at -20°C for 10 minutes. Cell debris was removed by centrifugation at 8000 rpm for 5 minutes at 4°C. Clear supernatant was transferred into a new tube. DNA was precipitated by adding equal volume of pre-chilled isopropanol and stored at room temperature for 5 min. DNA was collected by centrifugation at 8000 rpm for 5 min at 4°C. Supernatant was discarded and DNA pellet was washed with 400 µl of 70% ethanol. DNA pellet was air dried and re-suspended in purified water**.**

Agarose gel electrophoresis and DNA quantification: Quality of the DNA was observed on agarose gel. Agarose gel electrophoresis was performed as described by Sambrook *et al*. (2000). Quantity of DNA was measured using spectrophotometer (Bio-Rad) following the instruction manual of the manufacturer.

Cloning and sequencing of fungal ITS region: Genomic DNA of the selected fungal strains was used as a template to amplify ITS region by polymerase chain reaction (PCR). The PCR amplifications were performed with PCR *Pfu*-polymerase high fidelity enzymes (Tiangen) in Master cycler gradient (Eppendorf) with initial denaturation at 94°C for 3 min and 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 3 min each, followed by a 10 min final extension step at 72°C. Universal primers, ITS1 (TCCGTAGGTGAACCTGCGG), ITS4 (TCCTCC GCTTATTGATATGG) (White *et al.*, 1990) and ITS1-F (CTTGGTCATTTAGAGGAAGTAA) (Gardes and Bruns, 1993) were used for PCR amplifications. The ITS1 and ITS1-F acted as forward and ITS4 as reverse primer. PCR reaction was performed in 0.2 µL tubes containing 25 µL of reaction volume. PCR reaction mixture contained 0.2 µM of each of the primers (ITS 1 or ITS 1-F and ITS 4), 200 µM of dNTP's (deoxynucleotide phosphate), 2.0 units of *Pfu*-polymerase enzyme and 0.05 µg of template. The PCR products were purified using the QIA-quick gel extraction kit (Qiagen) and were stored at -20°C prior to ligation. The PCR amplicons were ligated into pTZ57R/T cloning vector (Fermentas, USA). Ligation reaction was performed as described by the instruction manual of Insta-Clone Kit (Fermentas, USA).

Recombinant vectors were transformed into *E. coli* Top-10 competent cells by heat shock (2 min, 42°C) method. Positive transformants containing desired inserts were selected on appearance of blue/white colonies after the incubation on LB (ampicillin) plates containing X-gal and IPTG. White colonies were picked and were further grown on LB medium supplemented with ampicillin (100µgmL^{-1}). Plasmid DNA was isolated from these cultures by using the Qiaprep miniprep kit (Qiagen, Valencia, CA) following the instruction manual of manufacturer. Purified plasmids containing our desired inserts (fungal ITS regions) were used for sequencing (Macrogen, Korea).

Identification of strains by BLAST: Fungal strains were identified on homology basis of their ITS regions, using BLAST (http://www.ncbi.nlm.nih.Gov/BLAST) at NCBI database. The sequences were submitted to (GenBank) (NCBI) data base.

RESULTS AND DISCUSSION

Fungal contamination is widespread in tropical countries, where poultry production and processing are spreading rapidly. The contamination of agricultural products with toxigenic fungi under favorable conditions leads to production of mycotoxins. As reported earlier, a total of 53 *Aspergillus* isolates were obtained from 119 samples of poultry feeds and out of these, 23 isolates, upon morphological basis were identified as *A. niger* aggregates (Saleemi *et al*., 2010).

Aspergillus species have been reported to produce different toxigenic substances including aflatoxins and ochratoxin A (Saleemi *et al*., 2010), cyclopiazoic acid (Duran *et al*., 2006), aspergillic acid, malformis (Andersen and Thrane, 2006), glutaconic acid (Lugauskas *et al*., 2005) and β-nitropropionic acid (Frisvad *et al.,* 2006).

Molecular methods, though expensive, laborious and time consuming, yet are considered as most authentic way of microbial identification and have become the most common tool for the identification of fungi in environmental samples (Borman *et al*., 2008). All the isolates exhibiting black color colonies were identified as *A. niger* aggregates (or black *Aspergilli*). Black *Aspergilli*, although morphologically similar, can further be identified as a variety of closely related species (Perrone *et al*., 2007). In present study, 12 isolates of *Aspergillus* species including five isolates with macroscopic characters suggestive of black *Aspergilli* were randomly selected from those isolated from the poultry feeds for further confirmation via cloning and sequencing the ITS (Internal transcribed spacer) region of the 5.8 S rRNA (ribosomal RNA). Apart from these, one randomly selected isolate of each of morphologically identified *A. flavus* and *P. chrysogenum* species was also used for

molecular identification. The genomic DNA (Fig. 1) was used as a template to amplify the ITS-region. The PCR amplicons (Fig. 2) were cloned and sequenced to identify the fungal isolate.

An un-rooted phylogenetic tree (Fig. 3) based on ITS sequences of fungal isolates was constructed using DNAMAN 6.0 program for identification. Identified fungal species along with their accession numbers (GenBank/DDJB) are given in Table 1. The ITS-regions contain most conserved sequence at the terminal region and also contain the hyper variable sequences distinguishing between species. Therefore, they have been considered as the best tool for the identification of the fungi. The use of ITS region as compared with other molecular probes is advantageous due to many reasons including increased sensitivity because of existence of more than 100 copies per genome (Mirhadi *et al.*, 2007).

On the basis of cloning and sequence analysis of *A. niger* aggregates, four isolates were identified as *A. tubingensis* and one as *A. niger*. Similar to our studies Martinez-Culebras and Ramon (2007) identified *A. tubingensis* as new species among black *Aspergilli* from Spain. Similarly, Moslem *et al*. (2010) identified ochratoxigenic black *Aspergilli* from coffee beans by molecular methods in Saudi Arabia. Identification of two different species in a group of five black *Aspergilli* suggested that *A. niger* aggregates or black *Aspergilli* should be further identified by molecular methods to differentiate between closely related species. Martinez-Cluebras and Ramon (2007) also suggested that phylogenetic analysis can be used to develop relationship between closely related species. Similarly, Chiotta *et al*. (2011) and Rodrigues *et al*. (2011) differentiated different toxigenic species in *Aspergillus* section *Nigri* by molecular characterization.

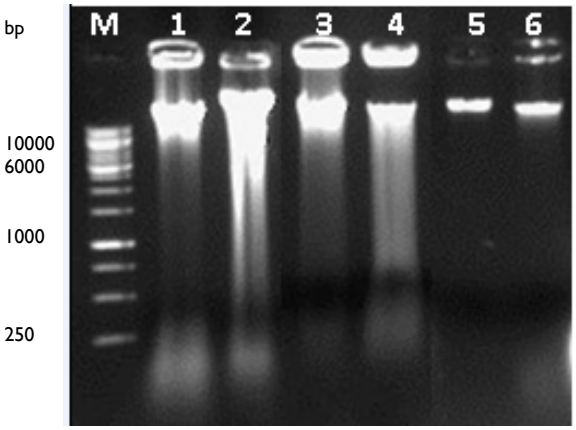

Fig. 1: Genomic DNA isolation of poultry feed fungal isolates. Fungal strains grown in Vogel Medium and mycelia harvested by centrifugation and genomic DNA extracted by Cenis method (Cenis, 1992). M= 1 kb DNA marker, Fermentas, Lane 1-6represent genomic DNA of different fugal strains on 1% agarose gel.

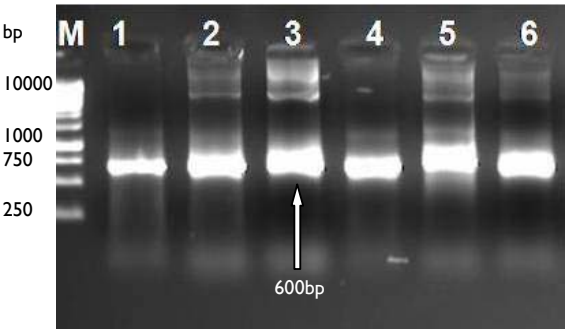

Fig. 2: PCR amplification of ITS regions from genomic DNA of fungal isolates. A 600 bp DNA fragment amplified using high fidelity *Pfu* DNA polymerase enzyme with ITS1 and ITS4 primer pair (ITS1 forward and ITS4 reverse primer), M= DNA marker (1 kb) Lane 1= UAF-15, Lane 2 = UAF-215, Lane 3=UAF-250, Lane 4=UAF-138, Lane 5=UAF-428, Lane 6=UAF-134.

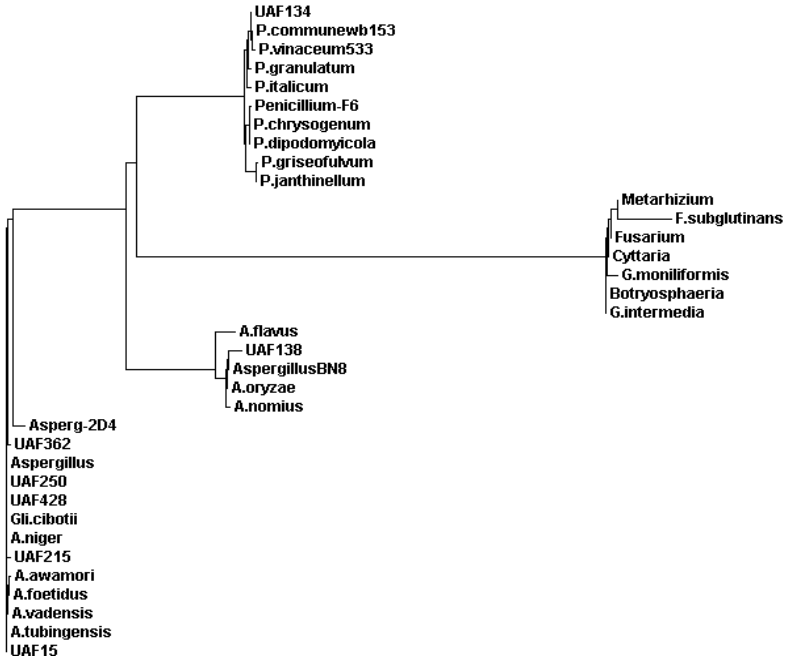

Fig. 3: An un-rooted phylogenetic tree of fungal isolates from poultry based on the ITS sequence homology. Tree was constructed using DNAMAN 6.0 by neighbor-joining method. Our isolates are indicated by shaded boxes.

Table 1: Identified fungal strains and their accession numbers

Species	Isolate	Accession No
A. tubingensis	215-UAF	FJ960823
A. tubingensis	250-UAF	FJ960824
A. niger	362-UAF	FJ960825
P. chrysogenum	134-UAF	FJ969192
A. flavus	138-UAF	FJ969193
A. tubingensis	428-UAF	GQ149491
A. tubingensis	15-UAF	GQ149492

Aspergillus flavus and *Penicillium chrysogenum* initially identified by morphological parameter appeared as the same species by molecular methods suggesting that morphological parameters were accurate and precise enough to identify these stains.

Conclusions: Molecular methods are the most reliable methods of identification and phylogenetic analyses of the poultry feed contaminating fungi. The genus *Aspergillus* is the most dominant mycotoxin producing poultry feed contaminant in Pakistan.

REFERENCES

Accensi F, J Cano, ML Abarca and FJ Cabanes, 1999. New PCR method to differentiate species in *Aspergillus niger* aggregate. FEMS Microbiol Lett, 180: 191-196.

Andersen B and U Thrane, 2006. Food-borne fungi in fruit and cereals and their production of mycotoxins, advances in food mycology. Adv Exp Med Biol, 571: 137-153.

Bhat R, RV Rai and AA Karim, 2010. Mycotoxins in food and feed: present status and future concerns. Comp Rev Food Sci Food Saf, 9: 57–81.

Bau M, MR Bragulat, ML Abarca, S Minguez and FJ Cabanes, 2005. Ochratoxigenic species from Spanish wine grapes. Int J Food Microbiol, 98: 125-130.

Borman AM, CJ Linton, SJ Miles and EM Johnson, 2008. Molecular identification of pathogenic fungi. J Antimicrob Chemother, 61: 7-12.

Cenis JL, 1992. Rapid Extraction of fungal DNA for PCR amplification. Nucleic Acid Res, 20: 2380.

Chiotta ML, MM Reynoso, AM Torres, M Combina and SN Chulze, 2011. Molecular characterization and toxigenic profile of *Aspergillus* section *Nigri* populations isolated from the main grape-growing regions in Argentina. J Applied Microbiol, 110: 445–454.

Duran RM, JW Cary and AM Calvo, 2006. Production of cyclopiazonic acid, aflatrem and aflatoxin by *Aspergillus flavus* is regulated by veA, a gene necessary for sclerotial formation. Appl Microbiol Biotechnol, 73: 1158-1168.

Frisvad JC, U Thrane, RA Samson and JI Pitt, 2006. Important mycotoxins and the fungi which produce them. Adv Exp Med Biol, 571: 3-31.

Gardes M and TD Bruns, 1993. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Mol Ecol, 2: 113-118.

Geiser DM, FM Harbinski and JW Taylor, 2000. Molecular and analytical tools for characterizing *Aspergillus* and *Penicillium* species at the intra- and intraspecific levels. In: Integration of modern taxonomic methods for Penicillium and Aspergillus classification (RA Samson and JI Pitt, eds). Harwood Academic Publishers, Gordon and Breach Publishing Group, Amsterdam.

Hanif NQ, M Naseem, S Khatoon and N Malik, 2006. Prevalence of mycotoxins in poultry rations. Pak J Sci Indus Res, 49: 120-124.

Lugauskas A, J Repeckiene and LH Novosinskas, 2005. Micomycetes, producers of toxins, detected on stored vegetables. Ann Agric Environ Med, 12: 253-260.

Martinez-Culebras, PV and D Ramon, 2007. An ITS-RFLP method to identify black *Aspergillus* isolates responsible for OTA contamination in grapes & wine. Int J Food Microbiol, 113: 147-153.

Mirhadi H, K Diba, P Kordbacheh, N Jalalizand and K Makimura, 2007. Identification of pathogenic *Aspergillus* species by PCR-restriction enzyme method. J Med Mycol, 56: 1568-1570.

Moslem MA, A Masraqi, KA Abd-Elsalam, AH Bahkali and MA Elnagaer, 2010. Molecular detection of ochratoxigenic *Aspergillus* species isolated from coffee beans in Saudi Arabia. Genet Mol Res, 9: 2292-2299.

Perrone G, A Susca, G Gozzi, K Ehrlich, J Varga, JC Frisvad, M Meijers, P Noonim, W Mahakarnchanakuls and RA Samson, 2007. Biodiversity of *Aspergillus* species in some important agricultural products. Stud Mycol, 59: 53-66.

Parenicova L, P Skouboe, RA Samson, L Rosen and J Visser, 2000. Molecular tools for the classification of black *Aspergilli*. In: Integration of Modern Taxonomic Methods of *Penicillium* and *Aspergillus* Classification; Samson RA and JI Pitt (eds); Harwood Academic Publishers, Amsterdam, The Netherlands, pp: 413-424.

Rodrigues P, C Santos, A Venâncio and N Lima, 2011. Species identification of *Aspergillus* section *Flavi* isolates from Portuguese almonds using phenotypic, including MALDI-TOF ICMS, and molecular approaches. J Appl Microbiol, 111: 877–892.

Sambrook J, P MacCalluon and D Russell, 2000. Molecular Cloning: A Laboratory Manual, 3rd Ed, Cold Spring Harbor Laboratory Press, New York USA.

Sage L, D Garon and FS Murandi, 2002. Fungal flora and ochratoxin A production in grapes and musts from France. J Agric Food Chem, 50: 1306-1311.

Saleemullaha, A Iqbal, IA Khalil and H Shah, 2006. Aflatoxin contents of stored and artificially inoculated cereals and nuts. J Food Chem, 98: 690-703.

Saleemi MK, MZ Khan, A Khan and I Javed, 2010. Mycoflora of poultry feeds and mycotoxins producing potential of *Aspergillus* species. Pak J Bot, 42: 427-434.

Taylor JW, DM Geiser, A Burt and V Koufopanou, 1999. The Evolutionary Biology and Population Genetics Underlying Fungal Strain Typing. Clin Microbiol Rev, 12: 126-146.

Varga J, JC Frisvad and RA Samson, 2009. A reappraisal of fungi producing aflatoxins. World Mycotoxin J, 2: 263-277.

Vogel HJ, 1956. A convenient growth medium for *Neurospora* (medium N). Microb Genet Bull, 13: 42-44.

White TJ, T Burn, S Lee and J Taylor, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Shinsky JJ, White TJ (eds). *PCR Protocols:* A Guide to Methods & Applications, Academic Press, San Diego, USA, pp: 315-322.

Zia MA, KU Rahman, MA Sheikh and IA Khan, 2010. Chemically treated strain improvement of *Aspergillus niger* for enhanced production of glucose oxidase. Int J Agric Biol, 12: 964–966.