Research in
ISSN : P-2409-0603, E-2409-9325
AGRICULTURE, LIVESTOCK and FISHERIES
An Open Access Peer Reviewed Journal

Open Access **Res. Agric. Livest. Fish.**
Research Article **Vol. 4, No. 3, December 2017: 165-171.**

INTERNAL TRANSCRIBED SPACER BASED IDENTIFICATION OF *Aspergillus fumigatus* ISOLATED FROM POULTRY FEED SAMPLES

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ARTICLE INFO

Received
11 December, 2017

Accepted
26 December, 2017

Online
31 December, 2017

Key words
Aspergillus fumigatus
ITS
ITS 1
ITS 2
Feed sample

ABSTRACT

Among over 180 *Aspergilli*, *Aspergillus fumigatus* is the most common etiological agent causing invasive mold infection mostly in immunocompromised human and animal. Besides, the fungus is used for various useful purposes. However, for the utilization of *A. fumigatus* as a useful candidate, accurate identification is crucial. Here, the research work was aimed at identifying *A. fumigatus* from poultry feed samples using conventional and molecular techniques. Out of 23 feed samples, 2 (8.7%) were found to be positive for *A. fumigatus*. The internal transcribed spacer 1 (ITS 1) and ITS 2 regions and the 5.8S ribosomal DNA (rDNA) region of the fungus were amplified by polymerase chain reaction. The ITS regions are located between the 18S and 28S rRNA genes, and rRNA gene for 5.8S RNA separates these two ITS regions. The isolated gene has been sequenced and deposited in the GenBank (accession no. KC142152). The gene was 100% similar to other reference species of *A. fumigatus*, whereas in phylogenetic analysis, a clear distance was found in the cases of other *Aspergilli*. Based on the unique nature of the ITS1 and ITS2 regions and phylogenetic analysis of the genes, *A. fumigatus* was correctly identified. The isolated strain could be a good candidate for further studies especially for utilization in the field of biotechnology.

To cite this article: Mahmud MM, J Hassan and KHMNH Nazir, 2017. Internal transcribed spacer based identification of *Aspergillus fumigatus* isolated from poultry feed samples. Res. Agric. Livest. Fish., 4 (3): 165-171.

INTRODUCTION

Aspergilli (singular: *Aspergillus*) are filamentous fungi which are ubiquitous in nature (Lasker, 2002; Rinyu et al., 1995). *Aspergillus fumigatus* is a well-known saprophytic fungus, typically found in soil and decaying organic matter, where it plays an essential role in carbon and nitrogen recycling process. Over the last two decades, *A. fumigatus* has become the most prevalent airborne fungal pathogen, causing severe and usually fatal invasive infections in immunocompromised hosts (Andriole, 1993; Beck-Sague´ et al., 1993; Bodey and Vartivarian, 1989; Denning, 1998; Dixon et al., 1996; Groll et al., 1996). *A. fumigatus* grown on certain building materials can produce genotoxic and cytotoxic mycotoxins, such as gliotoxin (Nieminen et al., 2002). Besides human, animals and birds are equally vulnerable to this organism causing a considerable economic loses (Sajid et al., 2006; Richard, 1991; Nazir et al., 2011). Aspergillosis in young chicks and pullets is commonly associated with overwhelming exposure to large numbers of conidia from heavily contaminated feed, litter, or the hatchery environment (Dyar et al*.,* 1984; Nazir et al., 2014)

Although *A. fumigatus* is the most common etiologic agent, being responsible for approximately 90% of human infections (Bodey and Vartivarian, 1989; Derouin, 1994; Kurup and Kumar, 1991), in recent years, the fungus has been used for various purposes, for example, decolonization of dye (Jin et al., 2007), bioremediation of toxic metal (Rao et al., 2005) and development of anti-cancer drug (Kato et al*.,* 2009). In the genome of this fungus, there are at least 79 cytochrome P450 monooxygenases (http://p450.riceblast.snu.ac.kr/species.php) and this group of enzymes is considered as versatile biocatalysts (Bernhardt, 2006; Nazir et al., 2010).

Generally identification of *Aspergilli* based on morphological methods requires adequate growth, usually of 4 days, for evaluation of colony characteristics and microscopic features. However, from more than 180 species of *Aspergilli* available, single species identification is very difficult and sometimes impossible. To overcome this problem, several molecular approaches have been used for the determination of *Aspergillus* from environmental and clinical samples (Bretagne et al., 1995; Einsele et al., 1997; Yamakami et al., 1996; Nazir et al., 2014). For the identification of *Aspergillus* at genus and/or species level, some targets are used such as 18S rRNA gene, mitochondrial DNA, and the intergenic spacer region. Also, use of ITS amplicons of different lengths by capillary electrophoresis was reported for identification of *Aspergillus* species (Turenne et al., 1999). Later, *Aspergillus* species were identified using Internal Transcribed Spacer (ITS) regions 1 and 2 (Henry et al., 2000; Nazir et al., 2014). The ITS regions are located between the 18S and 28S rRNA genes, and rRNA gene for 5.8S RNA separates these two ITS regions. The sequence variation of ITS regions has led to their use in phylogenetic studies of related organisms (Guarro et al., 1999). The objective of this study was to isolate and identify *A. fumigatus* from poultry feed samples, and subsequently confirmatory identification of *A. fumigatus* was done by analyzing the ITS 1 and 2 nucleotide sequences.

MATERIALS AND METHODS

Sample and culture

A total of 23 poultry feed samples were collected randomly from retail markets in Mymensingh, Bangladesh during January to April 2014, as described by Nazir et al. (2014). The feeds were treated as ready-to-serve. Ten grams of each sample was blended with 90 ml of distilled water and an aliquot inoculated in triplicate on to potato dextrose agar (PDA). The PDA plates were incubated at 30^{o}C for 7-10 days. Colonies representative of *A. fumigatus* were sub-cultured again on to PDA. Primary identification was made based on cultural, morphological and microscopic characteristics (Klich and Pitt, 1988). The fungus was sub-cultured on again on PDA. The plates were incubated at 30^{o}C in dark for another 10 days. Besides, the fungal isolate was also cultured on PDA slants, incubated at 30^{o}C for 10 days and stored at 4^{o}C in cold room for future studies. The frequency (percentage of samples in which *A. fumigatus* was present) was determined. Representative *A. fumigatus* strains were used for molecular and confirmatory identification.

Culture preparation and DNA extraction

Extraction of DNA from fungi was performed following the needle inoculation of 50 ml of Potato Dextrose (PD) broth (Difco Laboratories, Becton, Dickinson and Company, Sparks, MD 21152, USA) with conidia from a 7-day culture in PD agar and incubation under shaking condition (120 rpm) for 72 h at 30°C. The hyphae were recovered on a 0.45-mm-pore-size filter and washed with distilled water. Aliquots of the fungal hyphae were stored frozen at -80°C until use. The DNA was extracted following the method described previously (Nazir et al., 2014) with some modifications. In brief, prior to lysis, about 1 gm of hyphae was thawed and grinded in liquid nitrogen using pestle and mortar, which was then transferred to microcentrifuge tube (1.5 ml) and suspended in 660 µl of lysis buffer (50 mM Tris-HCl, 50 mM EDTA, 3% sodium dodecyl sulfate, 1% 2-mercaptoethanol). After vortexing, the microcentrifuge tube containing the grinded powder and buffer was incubated at 65°C in water bath for 1 h. After lysis, DNA was extracted using phenol-chloroform. The DNA was eluted with 300 µl distilled water and 1 µl of RNAse (100 mg/ml) was added and incubated at 65°C for 15 min. The purified DNA was stored at -20°C until used.

Primers

Two oligonucleotide fungal primers described previously (Henry et al., 2000) were used for amplification. The ITS region primers (ITS 1, 5´-TCC GTA GGT GAA CCT GCG G- 3´; ITS 4, 5´-TCC TCC GCT TAT TGA TAT G-3´) are the conserved regions of the 18S (ITS 1) and the 28S (ITS 4) rRNA genes that were used to amplify the intervening 5.8S gene and the ITS 1 and ITS 2 noncoding regions. Primers were purchased from the Cosmo Genetech Co, Ltd., South Korea.

PCR amplification and sequencing

The PCR assay was performed in a total reaction volume of 50 ml consisting of 5 µl of 10x PCR buffer (100 mM Tris-HCl [pH 9.0], 15 mM $MgCl_2$, 500 mM KCl, 1.0% Triton X-100); 0.2 mM dNTP (dATP, dCTP, dGTP, and dTTP) mix (SolGent Co., Ltd.); 20 pmole (each) primer; and 1.5 U of SolGent™ *Taq* DNA Polymerase (SolGent Co., Ltd.). Thirty five cycles of amplification were performed in AB Applied Biosystem Veriti 96 Well Thermal Cycler after initial denaturation of DNA at 95°C for 3 min. Each cycle consisted of a denaturation step at 95°C for 30 s, an annealing step at 50°C for 30 s, and an extension step at 72°C for 1 min, with a final extension at 72°C for 10 min following the last cycle. The PCR products were analyzed by electrophoresis on 1.5% (w/v) agarose gels stained with ethidium bromide (0.5 mg/ml). The products were purified using QIAquick® PCR Purification Kit (Qiagen). Using the ITS 1 and ITS 4 primers, the purified PCR products were directly sequenced by automatic DNA sequencer (ABI 3730XL; Applied Biosystem) using BigDye® Terminator v3.1Cycle Sequencing Kit following the instructions of the manufacturer.

Multiple alignment, phylogenetic analysis and confirmatory identification

Sequence comparisons of referenced strains and isolated *Aspergillus* listed in Figure 1 were made using ClustalX version 2.1, MEGA version 5.1 and Sequencher version 5.0 softwares. Sequences from referenced isolates were aligned to complete or partial ITS sequences available in GenBank after submission of sequence data from this study. Comparison of sequences from referenced isolates, feed sample isolate, and GenBank sequences was performed using a non-gapped, advanced BLAST search. Phylogenetic analysis based on neighbor joining of the sequences was determined using MEGA version 5.1 software (Nazir et al., 2010).

Based on multiple alignment and phylogenetic analysis, the fungus was identified confirmedly up to species level. The ITS sequences from related fungal strains and the accession numbers (in parenthesis) obtained from GenBank were used for analysis are as follows: *A. fumigatus* ATCC 1022 (HQ026746), *A. fumigatus* ATCC 16907 (AY214446), *A. fumigatus* ZH1 (JQ767180), *A. flavus* ATCC 16883 (AF138287), *A. niger* ATCC 16888 (AF138904), *A. terreus* ATCC 16792 (AF138290), *A. ustus* ATCC 201953 (AF157507) and *Emericella nidulans* ATCC 10074 (AF138289) (anamorph: *A. nidulans*).

Nucleotide sequence accession numbers

The sequence (18S-ITSI-5.8S-ITS2-28S region) of the isolate isolated from feed sample was deposited in GenBank (accession no. KC142152).

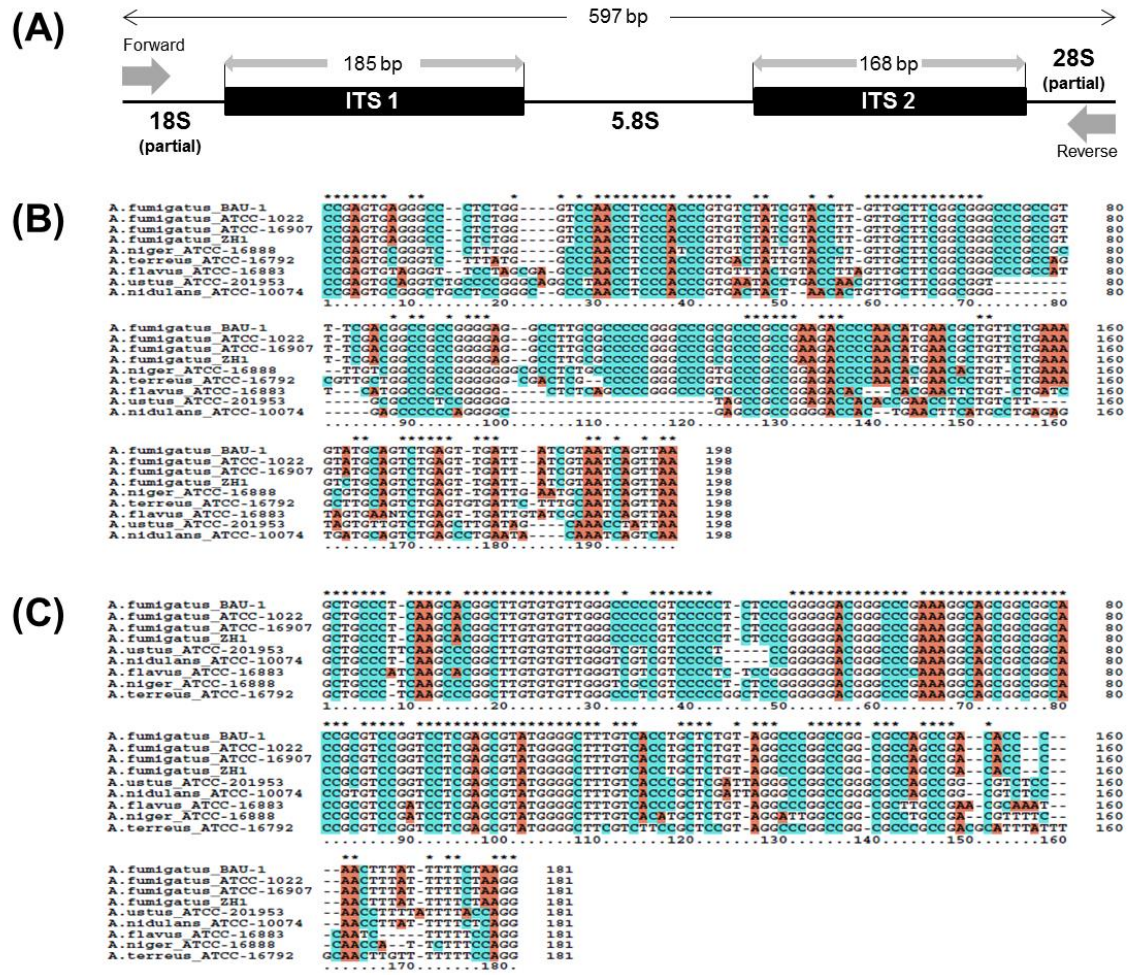

Figure 1. ITS-1 and ITS-2 Nucleotide sequence alignment of *A. fumigatus* BAU-1 (GenBank accession no. KC142152) and other intra- and interspecies fungal strains. (A) Diagram of sequence of 18S (partial), ITS-1, 5.8S, ITS-2 and 28S (partial). ITS-1 and ITS-2 are of 185 and 168 bp in size. (B) Nucleotide alignment of ITS-1 from BAU-1 stain with other related strains. (C) Nucleotide alignment of ITS-2 from BAU-1 strain with other related strains.
* indicates the conserved bases among the nucleotides.

RESULTS AND DISCUSSION

Isolation and identification of *Aspergillus fumigatus*

A total of 23 poultry feed samples were tested for isolating the *A. fumigatus*, of which 2 (8.7%) were found to be positive for *A. fumigatus* in culture, on the basis of colony characteristics. The colonies were velvety white at first, which turned to greenish-gray later. The findings of the present study were in line with the findings of Yokota et al. (2004) and Nazir et al. (2014). However, the incidence is higher than the results described by Azarakhsh et al. (2011), who found only 4.3% incidence of *A. fumigatus* in broiler feed indicating that the feeds were contaminated with fungal spore. The high incidence of *A. fumigatus* might be due to poor storing condition and unhygienic preparation, and its high adaptability to growth substrates in a wide range of environment and the production of spores (conidia) that remain viable even under extremely harsh conditions (Bardana, 1980). Inhalation of airborne conidia is the principal mode of exposure (Richard and Thurston, 1980). Once inhaled, the spores are deposited deep in the respiratory tract (Campbell, 1970).

Among the infectious diseases, fungal diseases have their own importance and seem to one of the great obstacle for the poultry farmers in the form of high morbidity, mortality and production losses (Sajid et al., 2006). The feed stuffs containing spores of *A. fumigatus* would act as a source of infection for poultry and subsequently a considerable economic loss may happen.

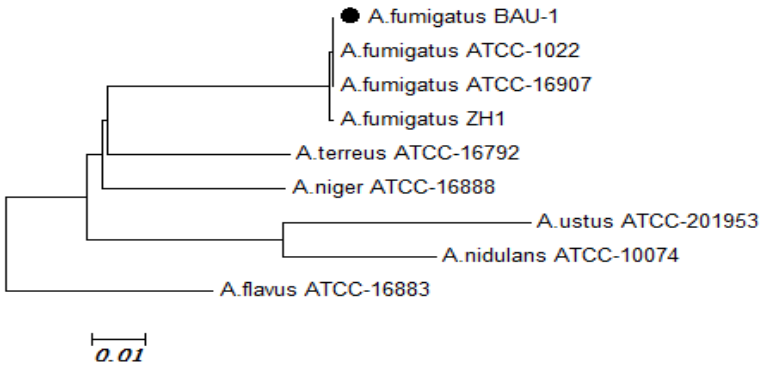

Figure 2. Phylogenetic analysis of BAU-1 strain with other intra- and interspecies candidates. The tree is showing that *A. fumigatus* BAU-1 strain is very close to the *A. fumigatus* ATCC 1022 and 16907 stains. Other strains are showing lesser or greater distance. Filled circle indicates our isolated strain.

Analysis of ITS 1-5.8S-ITS 2 regions and phylogenesis

Amplification of the ITS1-5.8S-ITS2 regions from the isolated *A. fumigatus* strains generated a PCR product size of 597 bp. After sequencing the regions, the nucleotide has been deposited in the GenBank (accession no. KC142152). More precisely, the regions contain a conserved region of 18S (partial), the variable regions of ITS 1 and ITS 2 separated by a conserved 5.8S region, and a relatively conserved 28S (partial) region (Figure 1) (Henry et al., 2000). Alignment of the gene sequences of our isolated *A. fumigatus* strain with other related fungi demonstrated that the 18S, 5.8S and 28S regions are remaining conserved, whereas ITS 1 and ITS 2 regions are differing with a varying degree (Einsele et al., 1997; Melchers et all., 1994; Van Burik et al., 1998). However, these two variable regions among the strains of *A. fumigatus* are showing 99-100% similarities. Based on the alignments, our isolated *A. fumigatus* strain is found to be 100% similar to *A. fumigatus* ATCC 1022 and *A. fumigatus* ATCC 16907. However, only one base pair is differing from *A. fumigatus* strain ZH1. On the other hand, considerable differences in gene similarities among interspecies observed (Figure 1); the sequenced region was 89, 92, 91, 93 and 93% similar to the corresponding region in *A. flavus* (ATCC 16883), *A. niger* (ATCC 16888), *A. terreus* (ATCC 16792), *A. ustus* (ATCC 201953) and *A. nidulans* (ATCC 10074), respectively, which supports the findings of Henry et al. (2000). Based on phylogenetic analysis, the isolated fungus is very similar to *A. fumigatus* ATCC 1022 and *A. fumigatus* ATCC 16907, whereas other fungi are placed to lesser or greater distance (Figure 2). After confirmatory identification of the fungus, the strain has been named as BAU-1, and kept at Bangladesh Agricultural University as a stock culture.

CONCLUSIONS

The results of this study showed that a considerable percentage of feed samples analyzed in this study are contaminated with *A. fumigatus*. A combined conventional and molecular approach was employed for accurate identification of *A. fumigatus*. The molecular approach was done by PCR amplification, sequencing and analysis of internal transcribed spacer (ITS) regions present in the genome. This findings warrant the need for analyzing the samples for *A. fumigatus* toxin and also to design effective management strategies to prevent contamination of feed to *Aspergillus* spores. The study highlights a potential risk of poultry as mentioned in title getting contaminated with hazardous toxic compound and potentially infective *A. fumigatus* in Bangladesh, thus making it for further analysis and continual monitoring and evaluation of feeds.

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