Morphological and molecular diagnosis of invasive aspergillosis in chickens

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Confirmatory diagnosis of invasive aspergillosis is paramount to ensure proper treatment and effective management of the disease in food and companion animals. Suspected invasive aspergillosis in chickens was encountered at post-mortem. Morphological and molecular methods were employed to identify Aspergillus from samples collected from dead chickens at post-mortem. Morphologically, two species of Aspergillus were identified, namely Aspergillus fumigatus and Aspergillus flavus. Molecular identification based on polymerase chain reaction (PCR) and sequence analysis of the partial 5.8 S rRNA, complete internal transcribed spacer-2 and partial 28S rRNA sequences bolstered morphological identification to arrive at the confirmatory diagnosis of the disease. Various hotspots that differentiate A. flavus from A. fumigatus and from other Aspergillus species were identified based on multiple sequence analysis. Maximum likelihood phylogenetic tree showed that isolates from the same species were grouped in the same clade. It is important to correctly identify the Aspergillus species in order to efficiently manage the disease.

Key words: Aspergillosis, chicken, morphology, molecular characterization.

INTRODUCTION

Aspergillosis is a severe fungal disease that affects a variety of domestic and wild birds that are kept in captivity. The most common etiologic agent is Aspergillus fumigatus but A. flavus, A. niger, A. nidulans, and other Aspergillus species or sometimes mixed infections can play a role in the disease (Barton et al., 1992; Perelman and Kuttin, 1992; Joseph, 2000; de Oca et al., 2017). The reason why A. fumigatus is the predominant species of airborne fungal infections might be that the spores are much smaller than the spores of other Aspergillus species (Richard and Thurston, 1983). Aspergillus species may be responsible for allergic bronchopulmonary disease, mycotic keratitis, otomycosis, nasal sinusitis, and invasive infection (Henry et al., 2000). Diagnosis of aspergillosis might be challenging because clinical signs of aspergillosis are non-specific, (Dahlhausen et al.,...
Figure 1. Visceral organs showing *Aspergillus* lesions. The arrows point to cream coloured nodules in the heart (A), peritoneum (B), airsacs, lungs and other visceral organs (C).

Figure 2. Co-infection of *Aspergillus flavus* (left) and *Aspergillus fumigatus* (right). From caseous nodules and heart lesions.

Therefore, diagnosis usually depends on a combination of evidence from the history, clinical presentation, and laboratory tests (Jones and Orosz, 2000). The fungi ribosomal RNA (rRNA) genes (rDNA) comprising small subunit (SSU) 18S rRNA, 5.8S rRNA, large subunit (LSU) 28S rRNA, and internal transcribed regions 1 and 2 (ITS1 and 2) (Khot et al., 2009) are the most universal target for their molecular identification. Most molecular detection and characterization of fungi are based on analyzing the ITS 1, ITS2 and the 5’end of the 28S gene (Fell et al., 2000; Abliz et al., 2004; Hinrikson et al., 2005; Walther et al., 2013; Trubiano et al., 2016, Gade et al., 2017). Moreover, Schoch et al. (2012) have proposed the ITS region as a universal barcode marker for fungi. This study seeks to use morphological and molecular methods to detect, identify and characterize the *Aspergillus* species involved in an infection observed in a poultry flock, and arrive at the confirmatory diagnosis of the disease.

**MATERIALS AND METHODS**

**Isolation and morphological identification**

Dead chickens were brought to the Poultry Unit of the Veterinary Teaching Hospital, University of Ibadan from a flock of 1000, and sixteen weeks old Issa Brown pullets with a morbidity rate of 40%. Post-mortem revealed presence of cream coloured nodules in internal organs of the chickens (Figure 1). Samples were aseptically taken from the nodules and heart lesions from the chickens and cultured on Sabouraud dextrose agar at 30°C for 7 days. Greenish and yellowish colonies were observed on the surface of the media (Figure 2). A small amount of the colonies was removed from the culture, stained with lactophenol cotton blue and observed under a biological microscope to study fungal morphology.

**Molecular identification and characterization**

Total DNA was extracted from isolated fungi using DNeasy Plant Mini Kit (QIAGEN, Valencia, California, USA) following manufacturers instruction. A pair of primers: ITS2F:5’-
A. fumigatus description of microscopic characteristics were in consonance with the arrangement (Figure 3). These macroscopic and curving parallel phialides in a columnar conidial colonies on the surface of the media. A. fumigatus on Sabouraud dextrose agar were greenish and yellowish features of fungi isolated from samples collected from the which was further confirmed with the colony and cellular velutinous blue-green colonies. Microscopically isolates were morphologically identified based on 18S rRNA-5.8S rRNA-ITS2 regions of the two Nigerian Aspergillus spp. The sequences have been deposited at the GenBank. These nucleotide sequences of the 18S rRNA-5.8S rRNA-ITS2 regions of the two Nigerian Aspergillus spp sequences retrieved from the GenBank and Cryptococcus neoformans var neoformans 18S rRNA-5.8S rRNA-ITS2 region as the outgroup was carried out. The multiple sequence alignment was carried out with clustal W analysis of multiple sequences from this study and seven sequences retrieved from the GenBank database using BLAST search via the National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple sequence alignment of the partial Aspergillus spp 18S rRNA-5.8S rRNA-ITS2 gene sequences from the two Nigerian Aspergillus spp sequences, other Aspergillus spp 18S rRNA-5.8S rRNA-ITS2 2 sequences retrieved from the GenBank and Cryptococcus neoformans var neoformans 18S rRNA-5.8S rRNA-ITS2 region as the outgroup was carried out. The multiple sequence alignment was carried out with clustal W algorithm in the CLC Main Workbench (Qiagen, Valencia, CA). Phylogenetic tree was generated using the maximum likelihood method coupled with the Kimura 2-parameter model with bootstrap analysis of 1000 replicates. Phylogenetic and molecular evolutionary analyses were conducted using MEGA, version 7.0 (Tamura et al., 2011).

RESULTS

History, sample collection, cultivation and microscopy

Affected birds presented for clinical diagnosis were grossly emaciated, and were too small in size for their age; indicative of a chronic condition typical of chronic aspergillosis. The numerous, cream coloured nodules observed at post-mortem are also typical of aspergillosis which was further confirmed with the colony and cellular features of fungi isolated from samples collected from the affected organs of the carcass. The fungal isolates grown on Sabouraud dextrose agar were greenish and yellowish colonies on the surface of the media. A. fumigatus isolates were morphologically identified based on velutinous blue-green colonies. Microscopically, A. fumigatus colonies possessed uniseriate conidial heads and curving parallel phialides in a columnar conidial arrangement (Figure 3). These macroscopic and microscopic characteristics were in consonance with the description of A. fumigatus by Kléch (2002). Aspergillus flavus isolates were morphologically identified based on yellow-green conidial colour, globose to sub-globose vesicles and biseriateserations. A. flavus colony also appeared compact and floccose. Staining with lactophenol cotton blue revealed spores of Aspergillus flavus and Aspergillusflavus (Figure 3).

PCR and sequence analysis

PCR amplification of the 5.8S rRNA-ITS-2-28S rRNA regions from the two aspergillosis samples generated PCR products of 350 bp. BLAST search revealed that sequence analysis carried out on the two sequenced amplicons were correspondent to A. fumigatus and A. flavus published sequences. The two were designated as A. fumigatus NGA1 and A. flavus NGA1 and have been deposited at the GenBank with SUB7514136 Seq1 MT533929 and SUB7514136 Seq2 MT533930 as the respective accession numbers. Multiple sequence alignments of the two nucleotide sequences from this study and seven sequences retrieved from the GenBank A. fumigatus MH91 (MH911420), A. fumigatus MN17 (MN178806), A. flavus MN18 (MN180857), A. flavus MK 13 (MK139781), A. niger AJ87 (AJ876876), A. nidulans NR13 (NR_133684) and A. terreus var. subflavus (A. terreus v NR14) (NR_149331) was carried out. As shown in Figure 4, at position 313 in the 5.8S rRNA gene adenine is substituted by cytosine in A. flavus. This substitution distinguishes A. flavus from other Aspergillus spp. In the ITS-2 gene, there is an insertion of adenine at position 394 in A. flavus which is absent in other Aspergillus spp analyzed. This insertion also differentiates A. flavus from other Aspergillus spp. At position 401, the presence of adenine in A. flavus and A. fumigatus differentiates them from A. niger, A. nidulans and A. terreus var. subflavus having cytosine at the same position. At position 450, cytosine is substituted for guanine in A. flavus thereby distinguishing it from other Aspergillus spp analyzed in this study. At position 537, adenine is unique to A. fumigatus. Also, at positions 555-557 nucleotides CTA are unique to A. fumigatus while in the 28S rRNA gene, at position 579 A. flavus and A. fumigatus possess adenosines.

The Nigerian Aspergillus sequences here analyzed is represented by the sequence of samples A. flavus NGA1 and A. fumigatus NGA1. In the 5.8S rRNA, position 313 distinguishes A. flavus, in ITS-2 positions 394 and 450 distinguishes A. flavus whereas position 401 distinguishes both A. flavus and A. fumigatus from other fungi. Also, in the ITS-2 gene positions 555-557 distinguishes A. fumigatus from other fungi. In the 5’ end of the 28S rRNA, position 620 distinguishes both A. flavus and A. fumigatus from other fungi sequences analyzed. All regions of mutations emphasized are in the boxes. Dots indicate position where the sequences analyzed are identical to that of the consensus sequence.

Phylogenetic tree was constructed via multiple
alignments of nucleotide sequence partial 5.8S rRNA, complete ITS-2 and partial 28S rRNA genes sequences and sequences retrieved from the GenBank (Figure 5). Cryptococcus neoformans var neoformans ITS-2 gene was used as the out-group. The tree was analyzed by maximum likelihood method with bootstrapping (1000). A. flavus and A. fumigatus clusters are labeled. Bar 0.05 nucleotide substitutions per site. A. flavus and A. fumigatus sequences from this study have Black Square and circle, respectively.

**DISCUSSION**

Conventional laboratory diagnosis of aspergillosis or other mycoses is usually based on morphological...
Figure 5. Phylogenetic analysis based on partial 5.8S rRNA, complete ITS-2 and partial 28S rRNA genes sequences.

characterization via direct examination or culture of the causative fungi. This approach is still necessary to categorize the isolates according to groups, which helps further identification by other methods (Zulkifli and Zakaria, 2017). In this study, morphological identification of Aspergillus spp obtained from a post-mortem case was carried out according to the method and species description by Klich (2002) and Samson et al. (2014), thereby identifying A. flavus and A. fumigatus. This identification method was bolstered by molecular characterization of the identified organisms to arrive at the confirmatory diagnosis of the disease. This approach is very important because certain Aspergillus spp are associated with higher mortality and increased virulence and vary in their resistance to antifungal therapy. A. fumigatus is the most common Aspergillus spp that causes invasive aspergillosis, although other species, such as A. flavus, A. niger, A. terreus, A. clavatus and A. nidulans, can also cause these diseases (van de Veerdonk et al., 2017). Based on morphological identification comprising microscopic and macroscopic methods, A. fumigatus colonies were greenish on the surface of the media whereas A. flavus were yellow-green. Microscopically, A. fumigatus colonies possessed uniseriate conidial heads whereas A. flavus were globose to sub-globose vesicles with biseriaterisations. Generally, macroscopic and microscopic characteristics such as colony colour and conidial shapes can be used to differentiate A. fumigatus from A. flavus.

The molecular detection of Aspergillus species from clinical samples has been achieved by amplification of parts of the rRNA region of fungi genome (White et al., 1990; Henry et al., 2000; Sabino et al., 2020). ITS1 and ITS2 have been employed in various phylogenetic studies of a variety of fungi. As such, these characteristics also make ITS regions reliable candidates for the identification of fungi at the genus or species level (Gaskell et al., 1997). Sequence analysis revealed positions that may be used to distinguish Aspergillus spp. such as A313C in 5.8S rRNA gene, an insertion of adenine at position 394 and G450C in ITS-2 gene. These three positions distinguished A. flavus from other Aspergillus spp analyzed. Also, C401A common to A. flavus and A. fumigatus differentiates them from A. niger, A. nidulans and A. terreus var. subfloccosus. In the 28S rRNA gene at position 579 A. flavus and A. fumigatus possess adenines which differentiate them from other Aspergillus spp analyzed. At positions 537 and 555-557 the presence of adenine and nucleotides CTA, respectively; are unique to A. fumigatus. Phylogenetic analysis further confirmed the identification of the Aspergilli to species level with A. flavus and A. fumigatus clustering in their respective clades. ITS1 and ITS2 have been employed in various phylogenetic studies of a variety of fungi. As such, these characteristics also make ITS regions reliable candidates for the identification of fungi at the genus or species level. It is generally believed that the ITS regions are more variable than 18S, 5.8S, or 28S rRNA genes. As mentioned above, we believe that highly species-specific sequences can be found in the ITS genes.

Conclusion

The study was able to identify the Aspergillus isolates
from a post-mortem case to specie level as *A. fumigatus* and *A. flavus* in a mixed infection, based on morphological identification and comparative sequence analysis of the 5.8 S-ITS2-18S rRNA regions.

**CONFLICT OF INTERESTS**

The authors have not declared any conflict of interest.

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