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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 aspergilllosis 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.,

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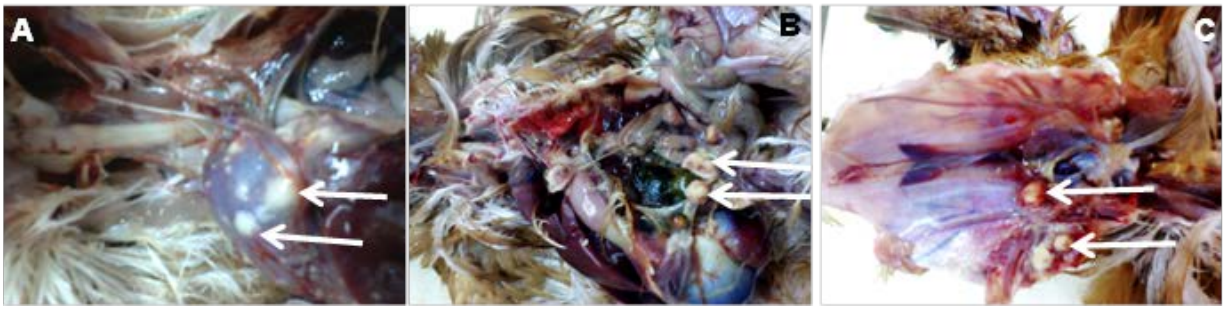

Figure 1. Visceral organs showing *Aspergillus* lesions. The arrows point to cream coloured nodules in the heart (A), peritoneum (B), airsacs, lungs and other visceral organs (C).

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

2004). 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 30ºC 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'-

GCATCGATGAAGAACGCAGC-3' and ITS2R: 5'-TCCTCCGCTTATTGATATGC-3' was used to amplify 350 bpsequence of 18S rRNA-5.8S rRNA-ITS2 genes (Gade et al., 2017). The PCR amplification reaction was carried out in 50 µl volume containing 5 µl of total DNA, 0.2 µM of each primer, 25 µl of PCR master mix (10 mMTris-HCl (pH 8.6), 50 mMKCl, 1.5 mM $MgCl_2$, 50 units/ml of Taq DNA polymerase, 0.2 mM each dNTP, 5% glycerol, 0.08% IGEPAL®CA-630, 0.05% Tween®20, 0.024% Orange G, 0.0025% Xylene Cyanol FF) and 18 µl of nuclease free water. The GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Foster City, CA) was used for amplification under the following conditions: 94°C for 2 min for initial denaturation, 35 cycles of 95°C for 20 s, 52°C for 1 min, 72°C for 1 min, and final extension at 72°C for 5 min. Two of the amplified DNA fragments from the samples were purified using GeneJET PCR Purification Kit (ThermoSCIENTIFIC®, Pittsburgh, PA). Automated nucleotide sequencing was performed on an ABI 3130XL. The sequences were edited with Sequence Scanner software, version 1.0 (Applied Biosystems, Foster City, CA) and designated *A. flavus* NGA1 and *A. fumigatus* NGA1. The sequences have been deposited at the GenBank. These nucleotide sequences of the 18S rRNA-5.8S rRNA-ITS2 regions of the two Nigerian *Aspergillusspp* sequences were compared with other published *Aspergillusspp* sequences already available in 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 *Aspergillusspp* 18S rRNA-5.8S rRNA-ITS2 gene sequences from the two Nigerian *Aspergillusspp* sequences, other *Aspergillusspp* 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, Valecia, 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 Klich (2002). *Aspergillus flavus* isolates were morphologically identified based on yellow-green conidial colour, globose to sub-globose vesicles and biseriateseriations. *A. flavus* colony also appeared compact and floccose. Staining with lactophenol cotton blue revealed spores of *Aspergillusflavus* and *Aspergillusfumigatus* (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. *subfloccosus* (*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. *subfloccosus* 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

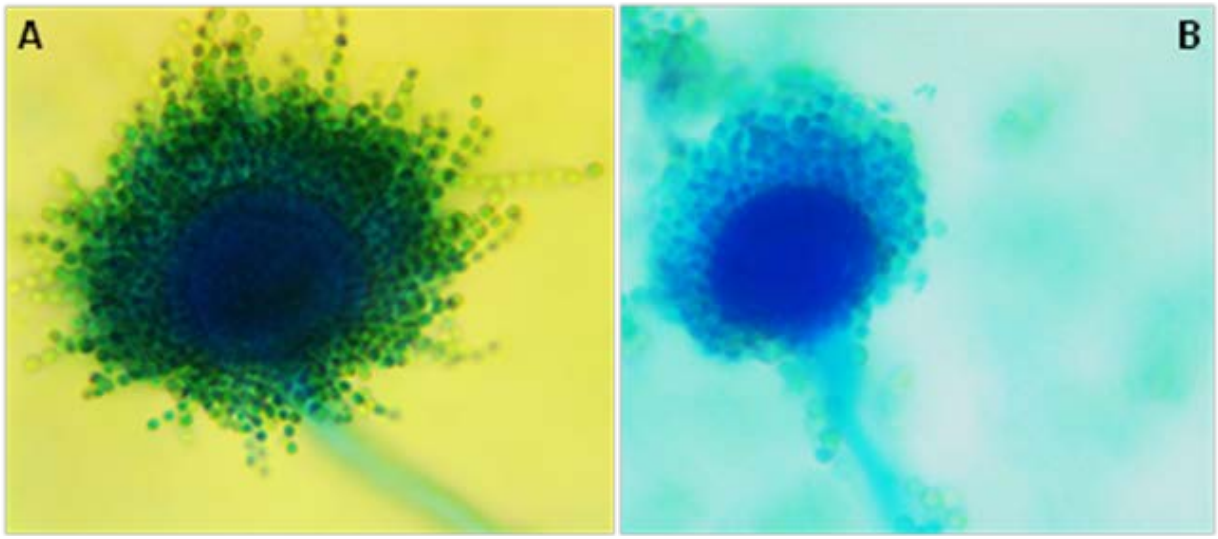

Figure 3. Light micrograph of *Aspergillusflavus* (A) and *Aspergillusfumigatus* (B) from caseous nodules and heart lesions. 40X magnification.

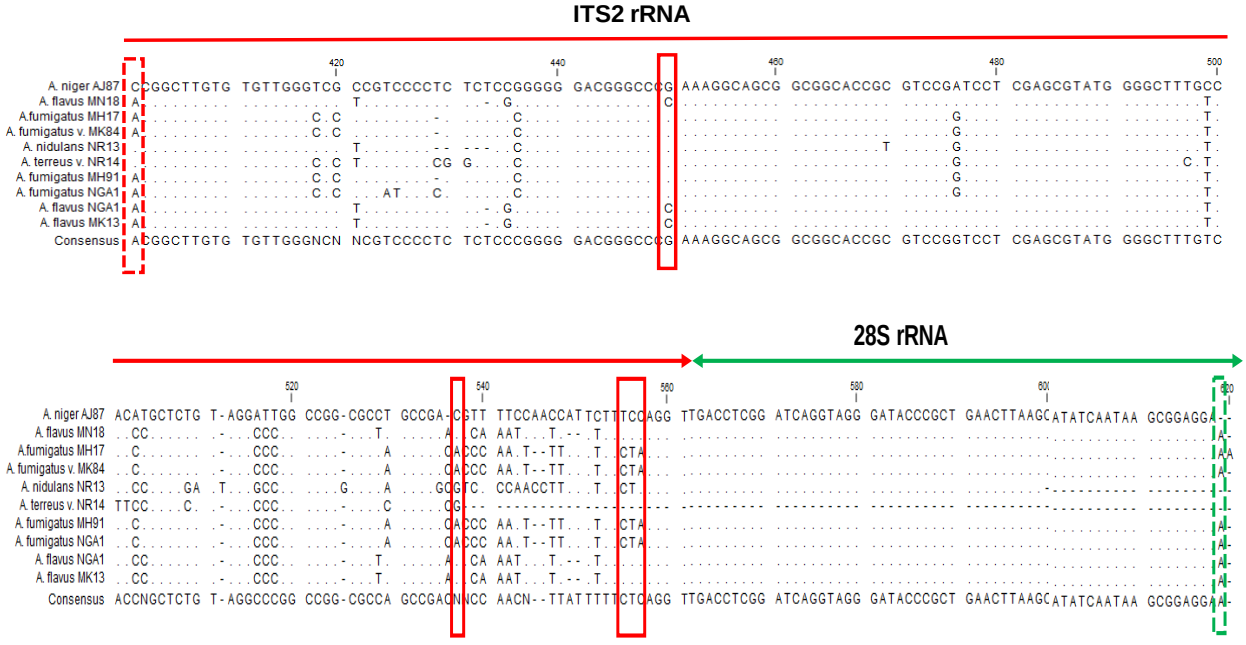

Figure 4. Multiple sequence alignment of the nucleotide sequences of partial 5.8S rRNA, complete ITS-2 and partial 28S rRNA genes of Aspergillusspp from this study and other fungi sequences retrieved from the GenBank.

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

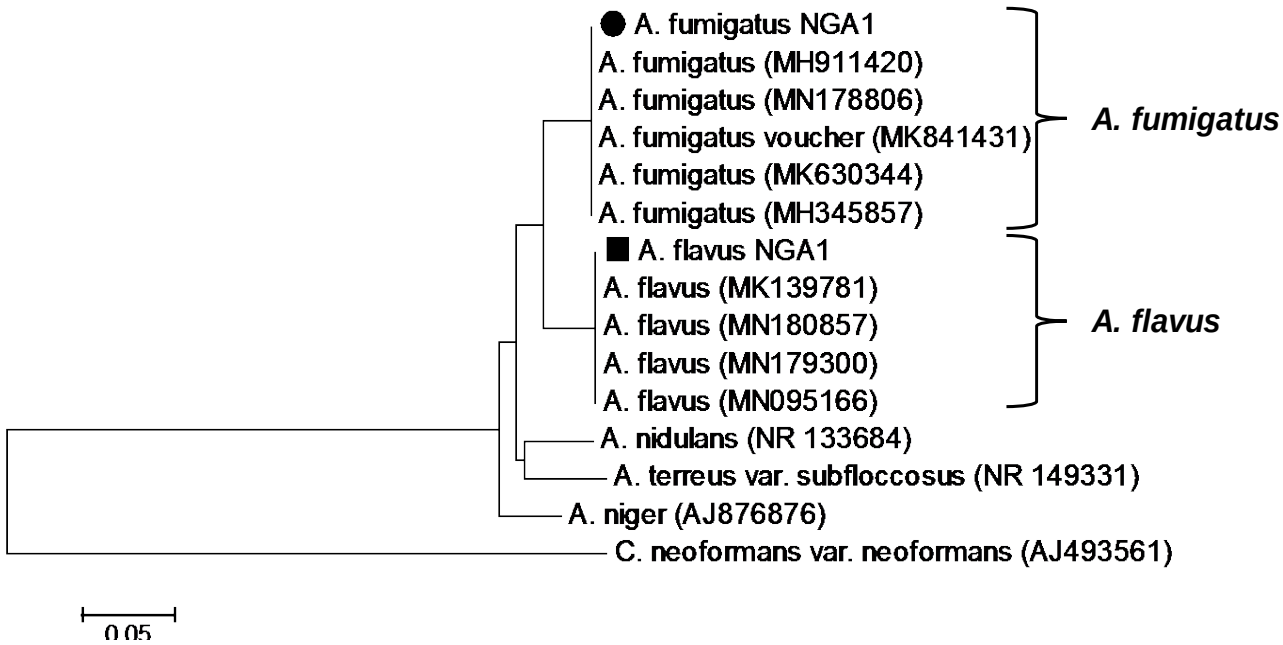

Figure 5. Phylogenetic analysis based on partial 5.8S rRNA, complete ITS-2 and partial 28S rRNA genes sequences.

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 biseriateseriations. 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 *Aspergillu*s 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 *Aspergillu s*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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