EXTENDED ABSTRACT – VAM 2010

IDENTIFICATION OF *Aspergillus flavus* FROM INTERNAL TRANSCRIBED SPACER REGIONS 1 AND 2 USING POLYMERASE CHAIN REACTION

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Abstract

Aspergillus flavus is identified as one of the most frequent causes of invasive aspergillosis in humans and animals. This organism is responsible for the elaboration of the toxin in the feed and the toxin is known as aflatoxin. There are various methods available to analyze aflatoxin in foods and feeds which can be classified as rapid, qualitative and quantitative methods. The aim of the project is to develop a rapid detection test kit for aflatoxin using Enzyme-labelled immunoassay technique. For this preliminary study, the primary goal is to produce aflatoxin antigen. In order to produce it, identification of the species available is necessary. Previously, this is usually done by morphological characteristics. Nowadays, a number of molecular, immunological and biochemical methods are available. In this study, the identification of *A. flavus* species, which is the main aflatoxin producer, was based on internal transcribed spacer (ITS) region using PCR methods. Previous studies indicated that, the ITS regions are located between the 18S and 28S rRNA genes and offer distinct advantages over other molecular targets including increased sensitivity due to the existence of approximately 100 copies per genome. By using this method, the identification of *A. flavus* was successfully conducted. In future, the toxin produced from this study can be used for the development of ELISA test kit for rapid aflatoxin detection. A quick and easy method for detecting aflatoxin in feed will help farmers to diagnose aflatoxicosis and treat it accordingly to minimise losses due to morbidity and mortality.

Keywords: *Aspergillus*, ITS Region 1 and 2, aspergillosis

INTRODUCTION

"Turkey X disease" was occurring in the 1960, which more than 100,000 young turkeys on poultry farms in England died. The syndrome was characterized by extensive liver damage including fatty change and subcutaneous haemorrhage. *Aspergillus flavus* and *Aspergillus parasiticus* were then identified as the organisms responsible for the elaboration of the toxin in the feed. The toxin is known as aflatoxin. There are four major types of aflatoxins called B_1, B_2, G_1, and G_2 have been characterize based on their fluorescence under UV light (which is blue and green) and relative chromatographic mobility during thin layer chomatograpy test (Bennett and Klich 2003). Aflatoxin is associated with both toxicity and carcinogenicity in human and animal populations. The diseases caused by aflatoxin consumption are known as aflatoxicoses, acute aflatoxicosis results in death and chronic aflatoxicosis results in cancer and immune suppression. The liver is the primary target organ, with liver damage occurring when poultry, fish, rodents, and nonhuman primates are fed aflatoxin B_1 (Bennett and Klich 2003).

Previously, identification of *Aspergillus* spp. usually done by morphological characteristics nowadays, a number of molecular, immunological and biochemical methods are available. Targets for the genus level detection of *Aspergillus* have included the 18S rRNA gene (Latha *et al.* 2008), mitochondrial DNA (Moody and Tyler 1990), the intergenic spacer region, and the internal transcribed spacer (ITS) regions (White *et al.* 1990). Internal transcribed spacer is widely used in taxonomy offer distinct advantages over other molecular targets including increased sensitivity due to the existence of approximately 100 copies per genome. These regions are located between the 18S and 28S rRNA genes.

MATERIALS & METHODS

Pure Culture Preparation and DNA extraction

Fungus isolated from animal feed positive for aflatoxin was grown on Sabouraud agar. Extraction of DNA was performed following the inoculation of 50 ml of Sabouraud dextrose (SAB) broth with conidia from a 7-day culture in SAB agar. Broth culture was incubated for 48 hours at room temperature. The hyphae were recovered by harvesting a loopful of colony from Sabouraud broth. The hyphae were washed with 70% sterile saline. Then, it was suspended in 400 µL of DNA extraction buffer (1 mM EDTA [pH 8.0], 1% sodium dodecyl sulphate, 10 mM Tris-HCl [pH 7.6], 100 mM NaCl, 2% Triton X-100) as mentioned by Van Burik *et al.* (1998). Microcentrifuge tubes containing hyphae and buffer was sonicated in a water bath for 15 minutes. Following that, the tubes were heated at 100°C for 5 minutes. Subsequent to lysis, DNA was purified using DNeasy ® Blood & Tissue kit (Qiagen). The protocols for crude cell lysates preparation were supplied by the manufacturer. The purified DNA was stored at -20°C until tested.

Primer

Two oligonucleotide fungal primers for ITS region were used for amplification as described by White *et al.* (1990): ITS 1, 5'- TCCGTAGGTGAACCTGCGG-3' and ITS 4, 5'- TCCTCCGCTTATTGATATG-3' make used of conserved regions of the 18S (ITS 1) and the 28S (ITS 4). These conserved regions of 18S and 28S are RNA genes which amplify the intervening 5.8S genes and the ITS 1 and ITS 2 non-coding regions (Henry *et al.* 2000). Primers were synthesized by the Medigene Sdn. Bhd.

DNA techniques

Amplification of ITS region was performed using *Taq* DNA polymerase (Invitrogen) for 40 cycles consisting of 30 second at 95°C (DNA denaturation), 30 second at 50°C (primer annealing) and 1 min at 72°C (DNA synthesis). The PCR products were purified using QIAquick® Gel Extraction Kit (Qiagen) accordance with the manufacturer's instruction.

RESULTS

A. flavus is characterised by the appearance of velvety texture, being white in colour before turning green after several days. Old culture became dark green and brownish after some time. *A. flavus* is viewed under 40X magnification on compound microscope The conidiophore is long, rough, pitted, spiny and have septae. This species has vesicle which produces conidia (asexual spores). Phialides point out in all direction and the conidia covers the entire vesicle or fruiting body. After several days, the colony turned green. Sabouraud broth became viscous. PCR reaction of ITS 1 and 2 was performed using genomic DNA from *A. flavus* as a template with the expected size of 500 to 750 bp was successfully amplified.

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DISCUSSION AND CONCLUSION

A. flavus appeared white in colour after overnight incubation, after 2 days, the white colony became green in colour. Reaching seven days of incubation, the colony turned dark green. After several weeks, it was developed into brownish colony. The velvety texture of *A. flavus* is due to the mass of hyphae overlapping each other and formed the mycelium. This fungus should be stained using lactophenol blue to maintain the structure and prevent the spores from dispersing to the air. This is crucial as the spores can cause respiratory infections to human and they can produce aflatoxin. When viewed under 40X magnification of compound microscope, it was showed vesicle that formed at the end of conidiophores. Vesicle produced conidia or asexual spores at the end of phialides. The conidiophores, which is the filamentous hyphae of *A. flavus* is septate, long and rough.

Gel electrophoresis is carried out to confirm the PCR amplification of the ITS of *A. flavus.* The band size is in between 500 to 750 bp. Further step for identification of this species should be done as this study only involved preliminary identification. It is suggested that sequencing of the PCR product of ITS regions should be performed to verify the identification further. The product that is successfully sequenced will be blast using NCBI.

ACKNOWLEDGMENT

The authors would like to thank the Bacteriology lab for their contributions and help in providing the fungus

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