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Original Article

Molecular Detection of Aspergillus Species from Food Sold in Amassoma, Nigeria

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ABSTRACT

Fungal infections are quite common in humans and range from common, superficial infections to invasive and life-threatening infection**s.** With at least 12 phyla and several fungal species globally, more than 500 species are expected to be infectious to man. One such is the ubiquitous Aspergillus species that mainly causes infection in immunocompromised individuals and those with underlying pulmonary disease. This study compared traditional identification methods and molecular biology techniques to isolate Aspergillus species from food samples sold in the University community. Some selected food samples frequently purchased in the community were analysed for the presence of Aspergillus species using both traditional and molecular biology techniques. Amplification of the ITS region of the rRNA genes of the isolate was performed using the ITS gene. A specie identified as *Aspergillus niger* was phenotypically identified as *Aspergillus sydowii*, and another species identified as *Aspergillus flavus* phenotypically was identified as *Aspergillus fumigatus* genotypically. The results demonstrate that traditional methods might be misleading, especially to a non-taxonomist. Genotypic identification proved to be more reliable and less time-consuming than phenotypic identification.

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INTRODUCTION

Fungal infections are quite common in humans and range from common, superficial infections to invasive, life-threatening infections involving the organs (Walsh et al., 1996). Most fungi exist in the environment, forming a portion of the normal flora in humans and animals (Pathakumari et al., 2020). Fungi are one of the most significant and common allergy producers, and it has been predicted that approximately 50% of people will develop allergic symptoms during their lifetime (Cordasco et al., 1995). Also, fungal infections contribute to increased morbidity and mortality in healthy and immunocompromised patients though it is most aggressive in the immunocompromised (Pathakumari et al., 2020).

With at least 12 phyla, there are several fungal species globally, with more than 500 species known to be infectious to man. One such is the Aspergillus species, a ubiquitous mould found indoors in homes and hospitals. It is also found in soil, seed, grains, and decaying vegetation. They are filamentous fungi and grow as saprophytes (Mousavi et al., 2016).

Aspergillus species secretes numerous mycotoxins into their environment, such as ochratoxin A, gliotoxin and aflatoxin (Pitt, 1994). Laboratory detection of fungi is achieved through conventional mycological methods and, more recently, proteomic and genomic approaches. Traditional techniques for detection require culture and isolation of the fungi. This simple but time-consuming method entails special mycological skills (Rodríguez et al., 2015). Thus, to overcome these limitations, many researchers have turned their attention to immunological and molecular-based assays that could enable early detection. In this study, we compared traditional identification techniques to molecular biology techniques to isolate Aspergillus species from food samples sold in the community.

MATERIALS AND METHODS

Study Area

The study was conducted at Niger Delta University, Amassoma, Bayelsa State. The samples were obtained randomly from the Amassoma community in Bayelsa State. Nigeria. The study area was chosen because it is a university community with students from different parts of the country.

Sample Collection

Four food samples frequently purchased in the community were used for the study: bread 1, Local bread 2, a week-old bread and tomatoes. Bread was chosen because it is a ready-to-eat food purchased by students and tomatoes are usually eaten with a local meat delicacy called SUYA.

Sample Processing and Identification

Mycological analysis of the food samples was performed by Hocking identification techniques (Hocking, 2006). The samples were serially diluted (1:10 dilution, 1 gm of food to 9 ml of sterile distilled water) and homogenised with a vortex mixer. 2 mls of each sample dilution was added 18 ml of Sabaroud dextrose agar in a tube and homogenised by a vortex mixer. The mixture was then poured into a petri dish. After 72 hrs of incubation, the plates were observed for the growth of fungal colonies. The samples were cultured on sabaroud dextrose agar and incubated at 37 ºC for 72 hours. The colonies were examined macroscopically and identified based on their colonial morphology, pigmentation, size, texture, and degree of opacity and also microscopically with the lactophenol blue test**.**

Microscopic examination

A drop of lactophenol blue was placed on a grease-free slide, and with a sterile wire loop, the fungal specimen was added to lactophenol blue

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and emulsified. A coverslip was placed over the suspension, and the edges were sealed with a sealant and examined microscopically.

Fungal Genomic DNA Extraction

Extraction was performed using a ZR fungal/bacterial DNA mini-prep extraction kit supplied by Inqaba, South Africa. The substantial growth of the pure culture of the fungal was suspended in 200 µl of isotonic buffer in a ZR bashing bead Lysis tube. 750 µl of lysis solution was put into the tube, secured in a bead beater fitted with a 2 ml tube holder assembly, and processed at maximum speed for 5 minutes. The ZR bashing bead lysis tube was centrifuged at 10,000xg for 1 minute.

400 µl of supernatant was moved to a Zymo-Spin IV spin filter in a collection tube and centrifuged at 7000 xg for 1 minute. To the filtrate, 1200 µl of fungal/bacterial DNA binding buffer was added to the collection tubes bringing the final volume to 1600 µl. Another 800 µl was pipetted to the Zymo-Spin IIC column in a collection tube and centrifuged at 10,000 xg for a minute. The flow-through was discarded from the collection tube after it was centrifuged. The residual volume was transferred to the same Zymo-spin and spun. DNA Pre-Was buffer (200 µl) was placed into a new Zymo-spin IIC collection tube and spun at 10,000xg for a minute, followed by 500 µl of the Wash Buffer and centrifuged at 10,000xg for another 1 minute.

The filtrate from the Zymo-spin IIC column was transferred to a clean 1.5 µl centrifuge tube, and another 100 µl of DNA elution buffer was added to the column matrix and centrifuged at 10,000xg. The ultra-pure DNA was then stored at -20 degrees for other downstream reactions.

Internal Transcribed Spacer (ITS) Amplification

Amplification of the ITS region of the rRNA genes of the isolate was performed using the ITS1 primers F: 5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITS4: 5'- TCCTCCGCTTATTGATATGC-3, on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 µl for 35 cycles. The PCR mix comprised the X2 Dream taq Master (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4 M and the DNA as a template. The PCR conditions were: Initial denaturation, 95 ^{o}C for 5 minutes; denaturation: 95^{o}C for 30 seconds, annealing: 53 ^{0}C for 30 seconds; extension: 72 ^{0}C for 30 seconds (35 cycles) and final extension: 72 ^{0}C for 5 minutes. The product was then resolved on a 1% agarose gel at 120V for 15 minutes and visualised on a UV transilluminator. All reagents used were supplied by mix supplied by Inqaba, South Africa

Sequencing of Products

Sequencing was performed by Inqaba Biotechnological, Pretoria, South Africa, using the BigDye Terminator kit on a 3510 ABI sequencer. The sequencing was achieved at a final volume of 10 ul; the components included 0.25 ul BigDye® terminator v1.1/v3.1, 2.25 ul of 5 x BigDye sequencing buffer, 10 uM Primer PCR primer, and 2-10ng PCR template per 100 bp. The sequencing conditions were 32 cycles at 96 °C for 10s, 55 °C for 5s and 60 °C for 4 min.

Blast Analysis

Obtained sequences were edited using the bioinformatics algorithm trace edit, and similar sequences were downloaded from the National Centre for Biotechnology Information (NCBI) database using BLASTN.

RESULTS

The colonial morphology and pigmentation of colonies identified on the samples after 72 hours on sabaraoud dextrose agar are shown in *Table 1* below**.** Aspergillus species were isolated from all samples isolated based on their colonial morphology.

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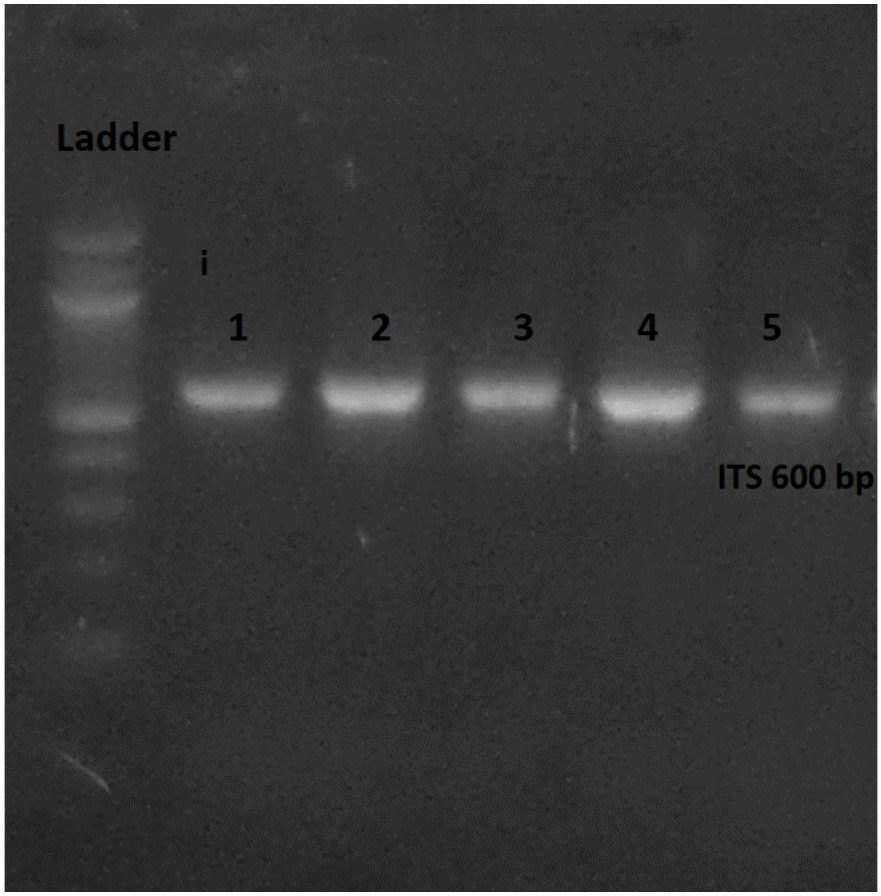
Table 1: Phenotypic characterisation of fungi isolated from the selected food samples

Sample	Pigmentation of colonies	Type of fungi
Bread 1	Black mould	Aspergillus
Bread 2	Yellowish green mould	Aspergillus
Tomatoes	Black mould	Aspergillus
Bread 3	Yellowish green.	Aspergillus

DNA extracted from the colonies was then resolved on the agarose gel with a 1000 bp ladder and ITS gene shown in *Plate 1*. The preferred DNA barcoding marker for the identification is the internal transcribed spacer of nuclear DNA

Plate 1: Agarose gel electrophoresis of the isolates



Bands on gel showing amplified ITS portion of the isolates from lanes 1-4 and while lane 5 represents the 100bp molecular ladder

The phenotypic result in *Table 1* above was compared with the genotypic result, and *Table 2* shows some discrepancies. *Table 3* displays the different species found in the food samples.

Table 2: Comparison of phenotypic and genotypic results

Phenotypic	Colour	Genotypic
Aspergillus niger	Black	*Aspergillus niger*
Aspergillus flavus	Yellowish green	*Aspergillus flavus*
Aspergillus niger	Black	*Aspergillus sydoweii*
Aspergillus flavus	Yellowish green	*Aspergillus fumigatus*
Aspergillus niger	Black	*Aspergillus niger*

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Table 3: Aspergillus species detected in the samples

Sample	Pigmentation of colonies	Aspergillus specie
Bread 1	Black mould	*Aspergillus niger*
Bread 2	Yellowish green mould	*Aspergillus fumigatus*
Tomatoes	Black mould	*Aspergillus sydoweii*
Bread 3	Yellowish green mould	*Aspergillus flavus*

DISCUSSION

Aspergillosis is a range of infections caused by the Aspergillus genus, and the species most involved include *A. fumigatus, A. terreus, A. flavus*, and *A. niger*. As stated earlier, the fungus mainly causes infection in immunocompromised individuals with underlying pulmonary disease and may be life-threatening, as seen in invasive pulmonary aspergillosis (Vuong et al., 2023). The high mortality linked with disseminated fungal infection has necessitated quickly identifying infectious moulds. The ITS regions have been used as targets because they generally display sequence variation between species and are used as an alternative to traditional methods.

Identifying most earlier Aspergillus species in cultures was the gold standard; *Aspergillus fumigatus* has been known for many years. Atypical forms of *A. fumigatus* have recently been re-identified as new species, including *A. lentulus*, *A. novofumigatus*, and *A. fumigatiaffinis*. These require DNA sequence analysis for reliable identification. (Bouzani et al., 2019)

Molecular identification using DNA barcoding has become an essential and integrated part of fungal research and provided new understandings of the variability of many different fungi groups (Belleman et al., 2010).

Aspergillus has several related species, and identification could be complex and tricky for a non-specialist trying to identify species within this genus. Mycologists have previously relied on categorising macroscopic and microscopic features, but more recently, identification to species level within a complex is easier done through molecular techniques (Balajee et al., 2009).

The prevalence of fungi in the environment and food makes them dangerous for humans because of the mycotoxins produced by these microorganisms. Realising that these Aspergillus species were found in ready-to-eat food raises a cause for public health concern.

The results of this study demonstrate that traditional methods might sometimes be misleading, as seen in Table 2. Results obtained genotypically were slightly different from the phenotypic identification. A specie identified as *Aspergillus niger* phenotypically was identified as *Aspergillus. Sydowii*, another specie identified as Aspergillus flavus phenotypically, was identified as *Aspergillus fumigatus* genotypically. This shows that the genotypic identification of fungi proved more reliable and less time-consuming than phenotypic identification.

16S and Internal Transcribed Spacer (ITS) ribosomal RNA (rRNA) sequencing are known sequencing techniques for the identification, identification and comparison of bacteria or fungi in the sample. This technique has been confirmed by different researchers (Janda and Abbott ., 2007; Manaka et al., 2017; Fida et al., 2021; Moreira et al., 2023). In this study, we used the Internal Transcribed Spacer ribosomal sequencing to identify Aspergillus species in selected food samples sold in a university community.

CONCLUSION

Due to phenotypic similarity, accurate identification of fungal species by conventional procedures remains tricky. In some cases, the etiological agent may often have been misidentified in previous literature. Molecular techniques have many applications in medical mycology. They are making an enormous impact on research globally, and in the future, their

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importance will increase in the detection, monitoring and identification of fungi. This will lead to better management and treatment of fungal diseases.

Recommendations

Molecular techniques have many applications in medical mycology, and as the technology keeps improving, mycologists should see it as a vital addition to traditional methods.

CONFLICT OF INTEREST

There are no conflicting interests

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