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Studies of food thickeners in Nigeria for contamination by aflatoxigenic forms of *Aspergillus* and their detection by PCR

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This paper reports the contamination of ready-to-use food thickeners, collected from the South-East geo-political zone in Nigeria, by aflatoxigenic form of *Aspergillus* species. A total of 150 samples from different open markets were observed for fungal contamination by using serial dilution-spread plate method. Although, *Aspergillus*, *Fusarium* and *Penicillium* were the most frequently isolated fungi, *Aspergillus* species were found to be the most prevalent in all the samples. Furthermore, *Aspergillus flavus* and *Aspergillus parasiticus* produced aflatoxin on yeast extract sucrose (YES) media incubated for 10 to 15 days at 27°C in a CO₂ incubator. *Aspergillus niger* showed no sign of any secondary metabolite on the media, set at similar conditions. Although, light microscopy was used to identify these fungi, based on colony morphology, PCR method was used to confirm genetic variation among the *Aspergillus* group, using ITS set of primers. Gel electrophoresis of PCR products confirmed the presence of *Aspergillus* species at an amplification range from 500 to 600 bp in all the samples tested. PCR was found to be a sensitive and a more reliable tool for detection and identification of *Aspergillus* species in food thickeners as opposed to conventional light microscopy. This is a first kind of mycological survey on the contamination of ready-to-use food thickeners sold in Nigeria.

Key words: *Aspergillus flavus*, *Aspergillus parasiticus*, aflatoxin, food thickeners, ITS.

INTRODUCTION

Food thickeners or thickening agents are used in food to absorb the fluid of the food without altering its physical or chemical properties. These are the modified food starch, polysaccharide or certain vegetable gums, which are used in beverages, gravies, sauces and stews. The use of food thickeners depends upon the type of food and purpose, for instance, some are used to increase taste, flavor, nutritive value, etc. These are good sources of nutrients such as carbohydrate, protein and fats. The protein content of food thickeners are compared to that of some important protein sources like oyster, pork and marine fishes (Lund et al., 2000; Ibrionke et al., 2006). A

wide range of thickening agents are available in Nigeria, which are mainly Achi (*Brachystegia eurycoma*), Akpalata (*Afzelia africana*), Ofor (*Detarium microcarpum*), and Ukpo (*Mucuna flagellipes*), and are commonly used by millions of Nigerians (Enwere, 1998). Currently, these are produced on a cottage industry scale, which varies from one ethnic group and locality to another. Practice of aseptic techniques during harvest, storage, transport, processing and packaging from the buying to selling point is minimal. The processing methods, in particular, vary from one site to another, thus, the microbiological quality of the products also varies.

Almost all the food or feed commodities can be contaminated by fungal organisms and many of the food- and feed-borne fungi are capable of producing one or more mycotoxins, particularly aflatoxins (Beatriz and Eliana, 2000; Achar et al., 2009). Aflatoxins produced by

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strains of *Aspergillus flavus* and *Aspergillus parasiticus* are hepatocarcinogenic, teratogenic and mutagenic, and have also been associated with growth stunting, underweight and modification of immune function in West African children (Gong et al., 2002; Turner et al., 2003). The degree of *Aspergillus* contamination and aflatoxin production is known to be influenced by many biotic and abiotic environmental factors (Molina and Giannuzzi, 2002). Fungal strain types, substrate, pH, temperature, relative humidity, moisture content of the substrate and aeration have been found to influence the quality and quantity of aflatoxin produced, in addition to the interaction between host and invading fungi (Chang et al., 2000). The data provided by Wild (1996) showed that about 98% of people in West Africa tested positive to aflatoxin biomarkers. At present, little or no data is known on fungal contamination of ready-to-use food thickeners sold to consumers in Nigeria. The non scientific methods of processing and poor handling of these agents are responsible for the contamination by aflatoxigenic form of *Aspergillus* species. Hence, there are possible health hazards associated with the consumption of such moldy food thickeners by Nigerian people. Furthermore, it has also not been possible to develop effective management strategies to prevent fungal infection and mycotoxin production. Hence, this study was undertaken to detect the fungal species associated with food thickeners in the South-East geo-political zone in Nigeria.

MATERIALS AND METHODS

Collection of samples

Ready-to-use food thickeners such as Achi, Akpalata, Ofor and Ukpo were obtained from different open markets located at Abia, Anambra, Ebonyi, Enugu and Imo states in Nigeria. From each state, 30 samples (a total of 150 samples) were collected with the aid of sterile polyethylene bags and transported to the laboratory aseptically for mycological evaluations.

Mycological analysis of food thickeners

All the samples were ground to a fine powder by using a dry coffee grinder (Toast Master, M1119). Each finely ground sample was serially diluted with saline, spread onto potato dextrose agar (PDA) plates and incubated at room temperature for 7 days. After the incubation period, the fungal colonies were observed under light microscope (Leica, M13595) and representative isolates of fungal colonies were transferred onto fresh PDA plates to study their macro- and micro-morphological characteristics. All the isolates were identified up to the species level by using fungal keys and manuals (Barnett and Hunter, 1972; Klich, 2002; Samson et al., 2004). The degree of fungal contamination was categorized as low (+), medium (++) and high (+++) for all the samples tested.

DNA extraction and PCR amplification of *Aspergillus* species

DNA was isolated using DNA isolation kit (MO BIO UltraClean Tissue & DNA Isolation Kit, CAT# 12334-50), following the

manual's instructions. The *ITS-1* (forward) and *ITS-4* (reverse) set of primers were used for PCR amplification (White et al., 1990). 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') made use of the conserved regions of the 18S (*ITS-1*) and the 28S (*ITS-4*) rRNA genes to amplify the intervening 5.8S gene with an expected amplicon size of 550 to 600 bp.

For PCR amplification of the genomic DNA, a PCR Kit (Epicentre Biotechnology FailSafe PCR kit) was used. The PCR was performed using 2.5 µl of DNA template, 0.5 µl of forward primer, 0.5 µl of reverse primer, 8.0 µl of deionized water, 1.0 µl of Taq Enzyme Mix and 12.5 µl of 2X PreMix A. The amplification reactions were performed in a total volume of 25 µl. The thermocycling profile consisted of 1 cycle of initial denaturation at 95°C for 1 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 65°C for 2 min, extension at 72°C for 2 min, followed by a final extension at 72°C for 5 min in the thermocycler model (Eppendorf). For electrophoresis, 15 µl of the PCR products was analyzed on 2.5% agarose gel, stained with ethidium bromide (BIO RAD's Mini Ready Agarose Gels, TBE# 161-3006), using 100 ml of 1 X TAE (Tris- acetate) buffer for 30 min at 100 V. A 1000 bp molecular marker was used for comparison. The gel was visualized and documented in the gel documentation system (BIORAD, Quant one 4.6).

Mycotoxicological analysis of *Aspergillus* species

Yeast extract sucrose (YES) agar was used in this study, as the medium for aflatoxin production, since YES has been reported to favor the production of high concentrations of aflatoxin (Gqaleni et al., 1997). All the isolated *Aspergillus* species were inoculated onto YES agar medium plates, sealed with parafilm and were incubated at temperature 27°C in a CO₂ incubator (Fischer Scientific, Isotemp) for 10 to 15 days. After the incubation period, plates were observed under ultraviolet light (Spectroline CC-80) to detect the presence of aflatoxin production (FenteFete et al., 2001). If the mold glowed under UV light, it was considered as aflatoxin positive and were categorized accordingly as aflatoxin formers and non formers.

RESULTS AND DISCUSSION

Fungi are well documented organisms that cause food spoilage and have always been a major concern for food experts because of their ability to produce mycotoxins. Fungal contamination makes the food unfit for consumption by causing discolouration, loss of nutrients, heating and mustiness (Bhattacharya and Raha, 2002). Fungi, especially those belonging to the genera *Aspergillus*, *Penicillium* and *Fusarium* have been reported to occur in food (Pitt et al., 1994; Gassen, 1999). Surveys conducted worldwide also revealed that *Aspergillus* species are known to frequently contaminate food and are able to produce mycotoxins such as aflatoxins, which are of serious problem (Wild and Hall, 2000; Achar et al., 2009). *Aspergillus* species are responsible for several disorders caused due to consumption of contaminated food and feed. Changes due to spoilage by *Aspergillus* species can be of sensorial, nutritional and qualitative nature such as pigmentation, discolouration, rotting, development of off-odors and off-flavors (Perrone et al., 2007). Furthermore, aflatoxigenic species of *A. flavus* Link and *A. parasiticus*

Table 1. Fungal contamination and presence of aflatoxin in ready-to-use food thickeners from open markets in South-East geo-political zone in Nigeria.

Names of food thickener and market		Name of fungi				
		<i>A. flavus</i>	<i>A. parasiticus</i>	<i>A. niger</i>	<i>Fusarium</i>	<i>Penicillium</i>
Food thickener	Commercial outlet*	Presence of toxin				
		(+ve)	(+ve)	(-ve)	(-ve)	(-ve)
Degree of contamination**						
Akpalata (<i>Afzelia africana</i>)	Onitsha market; Abakiliki market	+++	++	++	+	+
Offor (<i>Detarium microcarpum</i>)	Afor igwe-Ogidi market; Eke Awka market	+++	++	++	+	+
Achi (<i>Brachystegia eurycoma</i>)	Okigwe market; Owerri market	++	+++	+++	+	+
Ukpo (<i>Mucuna flagellipe</i>)	Umuahia market; Ogbete market in Enugu	++	+++	+++	+	+

*Samples collected from open markets in major cities and villages in Nigeria; **degree of contamination by fungi; +, degree of contamination considered as low; ++, degree of contamination considered as medium; +++, degree of contamination considered as high; (+ve), aflatoxin formers; (-ve) non-aflatoxin formers or no secondary metabolite.

Speare can produce potent carcinogen aflatoxin and can pose a significant human health threat (Reddy and Raghavender, 2007).

This study revealed the contamination of ready-to-use processed food thickeners by different fungi, namely: *Aspergillus*, *Fusarium* and *Penicillium*. The most common and prevalent fungi observed in all the samples tested were the *Aspergillus* species, namely *A. flavus*, *A. parasiticus* and *Aspergillus niger*. *A. flavus* was mostly common in Akpalata and Ofor samples, while *A. parasiticus* and *A. niger* were common in Achi and Ukpo. On the other hand, *Penicillium* and *Fusarium* species were observed in all the samples, however, to a lesser degree. The degree of contamination by each fungus varied from sample to sample (Table 1). These food thickeners, mainly found in the legume family, are usually processed into flour and used as soup thickener and stabilizer. In general, after processing into flour, the thickeners are spread on a mat or metal trays, for cooling before packaging in unsterilized bags. Moreover, in the open markets, unpackaged food thickeners are displayed in open basins or bowls for sale. With the surrounding environment, usually hot and humid, these practices are potential sources of fungal contamination, which may predispose the product to public health hazards (Perrone et al., 2007). This investigation confirmed that food thickeners were mainly contaminated by *A. flavus* and this condition may likely be due to mishandling, coupled by the prevailing environmental conditions from the packaging to the selling points. It is well known that *A. flavus* are common in tropical and temperate regions and are also found in desert, alpine and arctic areas, where harsh climatic conditions prevail (Perrone et al., 2007). *A. flavus* can be soil-borne, air-borne or carried in plant

residue, and can be recovered from any part of the plant from the deepest root to the highest flower. They are soil-borne because of their abundance in soil and their frequent association with plant roots, as either parasites or saprophytes. They are air-borne, because many have active or passive means of dispersal in the atmosphere and are the common colonizers of aerial plant parts. The widespread distribution of *A. flavus* may be attributed to the ability of these fungi to grow on a wide range of substrates and their efficient mechanisms for dispersal (Samson et al., 2004). Our study corroborates with these reports that *A. flavus* is ubiquitous in nature and that food thickeners provides another suitable substrate for the fungus to colonize and proliferate. High occurrence rate and distribution of moulds such as *A. niger* and *A. flavus* have been recorded in market garri samples (Ogiehor et al., 2007). These fungi have been implicated in some foods and beverages produced in Nigeria for human consumption (Ogiehor and Nwafor, 2004; Ohenhen et al., 2006).

The myco-toxicological study also confirmed that *A. flavus* and *A. parasiticus*, detected in the food thickeners, were of aflatoxin producing forms. Colonies of these two species glowed under UV light, on YES medium and were categorized as aflatoxin formers (+ve), while *A. niger*, showed no sign of any secondary metabolites, under UV, and was categorized as a non-aflatoxin formers (-ve) (Table 1). Since we established in this study, the contamination of food thickeners by fungi, with the high prevalence of *Aspergillus* species, especially the aflatoxigenic form, these ready-to-use food thickeners processed for sale to consumers in this region of Nigeria poses a public health hazard. Though the presence of *Aspergillus* species do not always indicate harmful levels of aflatoxin, however, the potential for aflatoxin

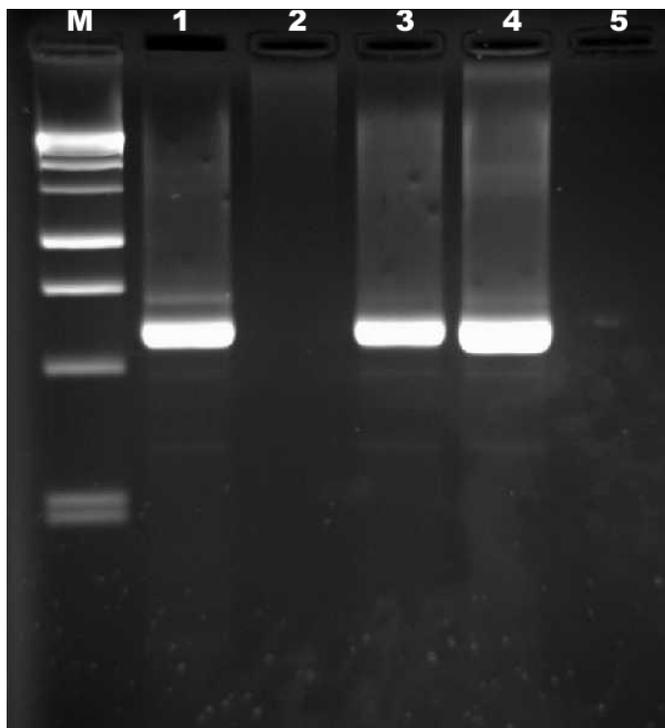


Figure 1. Agarose gel (2.5%), showing amplified products of ITS primers. Lane M, DNA marker; Lane 1, *A. flavus* from Akpalata; Lane 2, no DNA amplification; Lane 3, *A. flavus* from Offor; Lane 4, standard DNA of *A. flavus* for comparison; Lane 5, distilled H₂O as control.

production can be established. Exposures to aflatoxin through ingestion of contaminated foods and inhalation of toxins have been linked to acute and chronic toxicity in animals. Effects such as acute liver cirrhosis, induction of tumors and teratogenic and other genetic effects in animals and humans are well documented (Ibeh, 1994; Lund et al., 2000).

Early detection and characterization of potential aflatoxin-producing *Aspergillus* species is crucial in the prevention of toxins from entering the food chain. Detection of aflatoxin-producing fungal species by conventional methods is a labor- and time-consuming task that requires expertise. Molecular detection tools have been used to detect *Aspergillus* contamination in food thickeners. A diagnostic method based on the PCR is rapid, as there is no need to culture organisms prior to their identification. They are specific, since identification of species is made on the basis of genotypic differences, and are highly sensitive, detecting the target DNA molecules in complex mixtures, even when the mycelia are no longer viable (Xia and Achar, 2001; Somashekar et al., 2004; Sreenivasa et al., 2008). In our study, though *Aspergillus* species were more prevalent than either *Fusarium* or *Penicillium* in the samples tested, it was difficult to differentiate the *Aspergillus* group at the early stages of development by microscopic studies. Hence,

PCR was used to amplify the ribosomal DNA, using universal primers, internal transcribed spacer ITS 1 and ITS 4, and this confirmed the presence of *A. flavus*, *A. parasiticus* and *A. niger* in the different food thickeners tested. The results also confirmed that genetic variation between the three species (*A. flavus*, *A. parasiticus* and *A. niger*) was minimal with a common banding pattern ranging from 550 to 600 bp (Figures 1 and 2). ITS regions have been used successfully to generate specific primers capable of differentiating closely related fungal species (Bryan et al., 1995). The ribosomal RNA genes (rDNA) possess characteristics that are suitable for the detection of pathogens at the species level. These rDNA are highly stable and exhibit a mosaic of conserved and diverse regions within the genome (Hibbett, 1992). In this study, PCR using ITS primers, and in conjunction with light microscopy, confirmed the presence of 3 different species (*A. flavus*, *A. parasiticus* and *A. niger*) in food thickeners in this region of Nigeria.

Molecular technique such as PCR is known to be sensitive and reliable for detection and identification of fungal pathogens and hence we recommended the use of this tool for detection and confirmation of *Aspergillus* species in food thickeners in other geographic locations in Nigeria.

In conclusion, the ready-to-use food thickeners

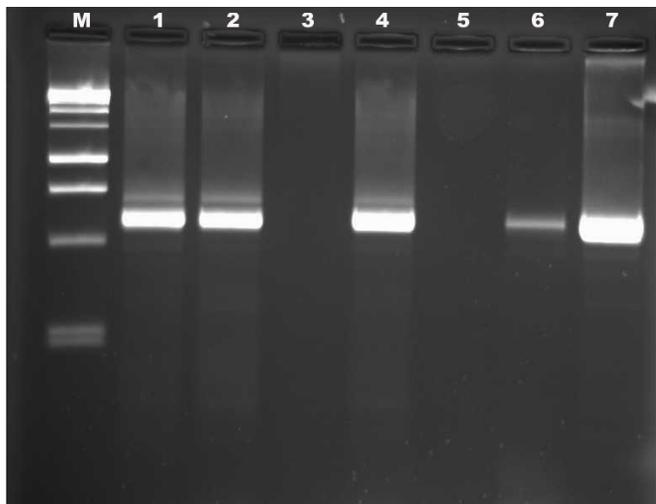


Figure 2. Agarose gel (2.5%) showing amplified products of ITS primers. Lane M, DNA marker; Lanes 1 and 2, template DNA of *A. parasiticus* from Achi and Ukp, respectively; Lane 3, no DNA amplification; Lane 4, *A. parasiticus* from Achi; Lane 5, distilled H₂O as control; Lane 6, *A. niger* from Ukp; Lane 7, standard DNA of *A. parasiticus* for comparison.

Available to consumers were contaminated with the aflatoxigenic form of *Aspergillus* species. If precautionary measures are not taken during harvest, storage and processing of these food thickeners from the buying and packaging to the selling point, contamination by *A. flavus* and *A. parasiticus* could raise serious concern related to environmental safety, food quality and human health. This is the first comprehensive report on the contamination of ready-to-use food thickeners in South-East geopolitical zone in Nigeria.

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