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Molecular Identification of Aflatoxigenic Fungi in Some Foods from Selected Markets in Lagos

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Author's contribution

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aflatoxigenic fungi are species of fungi that produce aflatoxins in food commodities. This study was aimed at screening different food samples in our local market for aflatoxigenic fungi using the aflatoxin regulatory gene (*aflR* gene). Six food samples (wheat, cowpea, rice, maize, melon and groundnut), were sourced from three different markets in Lagos metropolis (Mushin, Oyingbo and Mile 12). Fungi were isolated from these food samples and identified morphologically and microscopically. The genomic DNA was obtained using DNA isolation kits. The *aflR* gene was amplified from genomic DNA, nested, subjected to agarose gel electrophoresis and gel imaging. The Internal Transcribed Spacer (ITS) was also amplified from the genomic DNA for molecular identification of the organisms. The results showed that *Aspergillus flavus* were isolated from all the food samples from the three markets, while *Aspergillus niger* was present in rice, melon and wheat from Mile 12 market, maize and groundnut from Mushin market, rice and cowpea from Oyingbo market. *A. flavus* and *A.niger* were isolated from all the food samples when similar food samples from different market were mixed together. Only *A. flavus* amplicon from the nested polymerase chain reaction (PCR) showed approximately 400bp DNA fragment on the gel. This study has shown that PCR amplification of *aflR* gene has high specificity for detection of aflatoxigenic fungi in food samples thus, may be employed in screening food samples for contamination by aflatoxigenic fungi.

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Keywords: PCR; aflR gene; aspergillus flavus; aspergillus niger; Aflatoxin.

1. INTRODUCTION

Food is any nutritious substance that humans or animals ingest in order to maintain life and growth. Due to the importance of food to life, it should be safe from any form of contamination [1].

Nigeria is developing country facing many socio-economic challenges which include food insecurity and poor food safety [2]. The Food and Agriculture Organization [3] defines food security as a situation when all people, at all times, have physical, social, and economic access to sufficient, safe, and food that are nutritious for their dietary needs and food preferences for an active and healthy life.

There has been great concern about unsafe foods especially among low income earners. Certain microorganisms have been attributed to food poisoning and food toxicity [4].

More often, only few traceable contaminants, such as aflatoxins, form part of many microorganisms' toxins, which contaminate foods in storage or before storage, when the conditions are favorable [5, 6].

Aflatoxins are potent carcinogenic and mutagenic secondary metabolites produced by several *Aspergillus* species. Aflatoxins contamination in food pose serious problem as they have adverse effects on animal and human health thereby causing economic problems for international trade particularly in developing countries. Several food crops such as maize (*Zea mays*), peanut (*Arachis hypogea* L.), sorghum (*Sorghum bicolour*), and tree nuts are susceptible to contamination by aflatoxigenic fungi (*Aspergillus flavus* and *A. parasiticus*) [7].

One of the methods of detecting aflatoxins in food samples is to identify the presence of aflatoxinogenic fungal in the sample. Several techniques such as traditional plating, immunological and diagnostic methodologies can be used for the detection but each method has its limitations [8, 9]. For instance, the traditional plating method consumes time, requires some technical know-how and it is labor intensive. The immunological and diagnostics methods on other hand require several purification steps and are insensitive in the detection of false positives aflatoxinogenic fungi. To this reason, the development of an efficient, versatile, non-radioactive probes, time saving, cost effective and sensitive method becomes imperative as compared with the above methods.

Chen et al. [10] reported the development of a PCR-based method to identify aflatoxigenic fungi from peanuts. Shapira et al. [11] in previous study used standard PCR to detect aflatoxigenic fungi in grains. Farber et al. [12] collaborated in a study to perform a monomeric PCR to detect aflatoxin-producing strains in contaminated figs.

Aflatoxins regulatory gene (*aflR*) provides a useful amplification target that can be used to screen for those organisms that biosynthesize aflatoxins. The aim of the study is to show that PCR amplification of *aflR* gene has high specificity for detection of aflatoxigenic fungi in food samples thus, may be employed in screening food samples for contamination by aflatoxigenic fungi in Nigeria.

2. MATERIALS AND METHODS

All materials and reagents used in this study were of analytical grade and were sourced from standard chemical companies.

2.1 Collection of Raw Food Samples from Markets in Lagos

Raw food samples (Groundnut, Rice, Wheat, Maize, Cowpea and Melon) were randomly collected from three (3) different markets (Mushin, Mile 12 and Oyingbo) Lagos metropolis. The samples were ground and sieved and stored in sterile container for further use.

2.2 Isolation of Fungi from Food Samples

Culturing of food samples was done using potato dextrose broth (PDB) and potato dextrose agar (PDA). The PDB was prepared by weighing 200g of potatoes, boiled in 1 liter of distilled water for 30 minutes and then mashed. The mashed potatoes were filtered using muslin cloth. The filtrate was saved and used in the preparation of the agar. The filtrate was made up to 1 litre and 20g of dextrose was added. The preparation was autoclaved for 15mins at 121°C. Thereafter, 1g of each food sample was mixed with 10ml of each broth in a sterilized test tube and then 2ml of the mixture was added to 100ml of potato dextrose broth in a conical flask and incubated at 30°C for 24 - 72 hours [13].

2.3 Fungal Identification

The fungal isolates from the mixed food samples was first identified morphologically on plate and then viewed under a light microscope magnifying the fungi by 40. Both the mycelia and the spore of the fungi was carefully scrapped and placed on micoslide using a sterile surgical blade, a drop of methylene blue was added and a cover glass was placed and then viewed under the microscope [14]

2.4 DNA Isolation from Fungal Cultures in Mixed Food Samples

Similar food samples from different markets were mixed and pure fungal isolates obtained following the above procedure was on PDB. The different fungal isolate obtained were propagated on Potato Dextrose Agar (PDA). PDA was prepared by weighing 200g of Irish potatoes were weighed, peeled, sliced and boiled in 1 litre of distilled water for 30 minutes and then mashed. Filtration was done using muslin bag, the filtrate was made up to 1 litre with distilled water. 20g of dextrose (Glucose-D) powder was added and stirred with a stirrer. 20g of agar powder was added and autoclaved for 15 minutes at 121°C. 20 ml portions were dispensed into sterile 15×100mm petri dishes and incubated at 30°C for 24 - 72 hours [13].

The spores and mycelia of each fungus were scrapped carefully into eppendorf tube for DNA isolation using the Animal and Fungi DNA Preparation kit (Jena Bioscience, Jena Germany), following the manufacturer's instructions.

2.5 Primers and PCR Chemicals

Polymerase chain reaction was used to amplify the aflatoxin regulatory gene fragments and the ITS region of the fungal genomic DNA.

The sequence of the forward and reverse primers *aflR1* of the aflatoxin regulatory gene was (5'-AACCGCATCCACAATCTCAT-'3) and (5'-AGTGCAGTTCGCTCAGAACA-'3) and also the sequence of the forward and reverse primers a*flR2* of the aflatoxin regulatory gene was (5'-GCACCCTGTCTTCCCTAACA-'3) and (5'ACGACCATGCTCAGCAAGTA-'3) were ordered as custom primers from Inqaba Biotechnical Industries (South Africa), based on the published sequence strand for *Aspergillus flavus* and *Aspergillus parasiticus* (Accession No. 264763) [15]. The sequence of the forward (ITS1) and reverse (ITS4) primers of the Internal Transcribed Spacer was (5'-TCCGTAGGTGAACCTGCGG-'3) and (5'-TCCTCCGCTTATGATATGC-'3) were ordered as custom primers from Inqaba Biotechnical Industries (South Africa) according to White et al.,[16].

2.6 Preparation of PCR Master Mix

PCR master mix was prepared with: PCR grade water- 130µl, 2x Taq master mix- 250µl, Forward and reverse primers-10µl each. The resulting master mix 20 µl was added in a PCR tube and then 5µl of DNA template was added.

2.7 Amplification of ITS Gene

The polymerase chain reaction was performed in 25µl reaction mixtures containing 20µl 0f the PCR master mix described above and 5µl of the DNA template. Each reaction mixture was heated to 94ºC for 5 minutes. A total of 30 PCR cycles, each cycle at 0.3 min at 94ºC for denaturation, 0.45 min at 50ºC for annealing, 1.15 min at 72ºC for extension and a 10 min final extension at 72ºC was run on a programmable DNA thermal cycler [17].

2.8 Amplification of *aflR* Gene

The aflatoxin regulatory gene (*aflR*) was amplified from the genomic DNA of fungal isolates by polymerase chain reaction (PCR) technique using the forward primer of *aflR1* gene (5'-AACCGCATCCACAATCTCAT-'3) and the reverse primer of *aflR1* gene (5'-AGTGCAGTTCGCTCAGAACA-'3). The polymerase chain reaction was performed in 25 µl reaction mixtures containing 20µl 0f the PCR master mix described above and 5µl of the DNA template. Each reaction mixture was heated to 94ºC for 5 minutes. A total of 30 PCR cycles, each cycle at 0.3 min at 94ºC for denaturation, 0.45 min at 50ºC for annealing, 1.15 min at 72ºC for extension and a 10 min final extension at 72ºC was run on a programmable DNA thermal cycler [17].

2.9 Nested PCR

Nested PCR was done using the amplicons from first round of PCR amplification above as the DNA template with the forward primer of *aflR2* gene (5'-GCACCCTGTCTTCCCTAACA-'3) and the reverse primer of *aflR2* gene (5'-

ACGACCATGCTCAGCAAGTA-'3). A total of 30 PCR cycles, each cycle at 0.3 min at 94ºC for denaturation, 0.45 min at 50ºC for annealing, 1.15 min at 72ºC for extension and a 10 min final extension at 72ºC was run on a programmable DNA thermal cycler [15].

The amplicons were run in an electrophoretic setup using 1.5% agarose gel in TAE buffer at 100V for 25-30 minutes. EZ-Vison In-Gel stain was used for visualization. Loading dye was mixed with amplicons before gel loading. Photographs of gel were taken using Invitrogen safe Imager and analyzed to make reasonable conclusions.

3. RESULTS

3.1 Fungal Isolates from the Food Samples and Identification

Ground food samples such as Groundnut, Melon, Wheat, Rice, Cowpea and Maize were cultured in PDB for fungal isolation. Results obtained showed that most isolates had wooly colonies and the colour of colonies was olive green on PDA plate (Fig. 1). The photomicrograph showed that the conidia were round with finely roughened wall with hypal structure and was tentatively identified as *Aspergillus flavus* . An isolate with a thread appearance and soft texture was obtained, surface colony colour was initially white but turns to black. The photomicrograph showed a black globose conidial head with hypae. These characteristics conform to that of *Aspergillus niger*, hence the fungus was tentatively identified as *A. niger* (Fig. 1).

A. flavus had higher frequency of occurrence food samples obtained from the three markets in Lagos (Table 1).

Similarly, *A.flavus* and *A.niger* were the isolates obtained from all the different food types when similar food samples from the three markets were mixed (Table 2). The macroscopic morphology of the fungal isolates from the mixed food samples on plate after 72 hours is shown in Fig. 2.

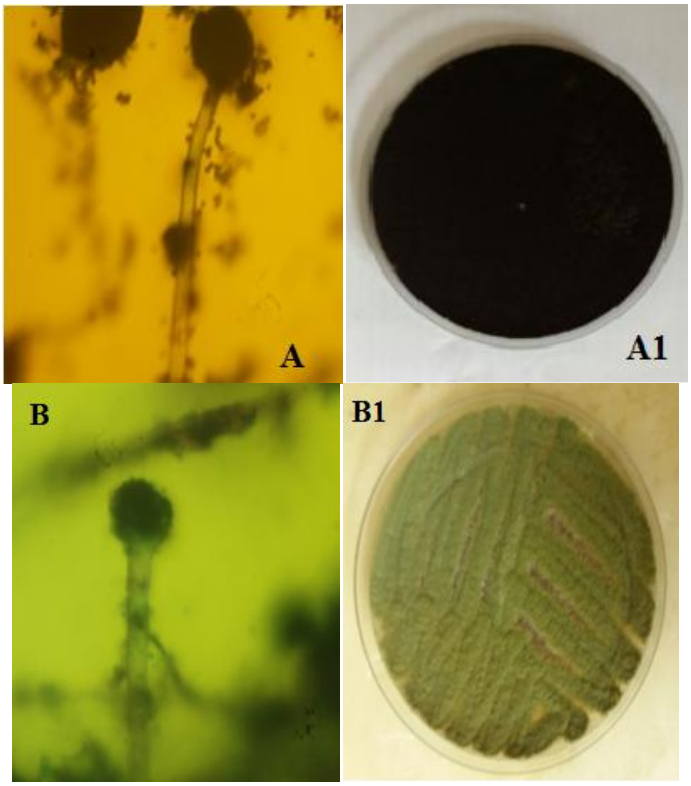

Fig. 1 Microscopic feature of *A.flavus* and *A.niger* (40 X) and Three days Growth of *A. flavus* and *A.niger* on PDA

KEYS: (A) Microscopic view of Asperillus niger, (A1) Plate morphology of Asperillus niger (B) Microscopic view of Aspergillus niger (B1) Plate morphology of Aspergillus flavus

Table 1. Fungi *Aspergillus* isolated from each food samples purchased from three selected markets in Lagos

Food samples	Markets	Fungal isolates				
		A.flavus	***A.parasiticus***	***A.niger***	***Penicillium***	***Fusarium***
Maize	Mile 12	+	-	-	-	-
	Mushin	+	-	-	-	-
	Oyingbo	+	-	+	-	-
Groundnut	Mile 12	+	-	-	-	-
	Mushin	+	-	+	-	-
	Oyingbo	+	-	-	-	-
Melon	Mile 12	+	-	+	-	-
	Mushin	+	-	-	-	-
	Oyingbo	+	-	-	-	-
Wheat	Mile 12	+	-	+	-	-
	Mushin	+	-	-	-	-
	Oyingbo	+	-	-	-	-
Cowpea	Mile 12	+	-	-	-	-
	Mushin	+	-	-	-	-
	Oyingbo	+	-	+	-	-
Rice	Mile 12	+	-	+	-	-
	Mushin	+	-	-	-	-
	Oyingbo	+	-	+	-	-

Keys: (+) = present; (-) = absent

Table 2. Fungi isolated from mixed the food samples

Food samples	***A.flavus***	***A.parasiticus***	***A.niger***	***Penicillium***	***Fusarium***
Maize	+	-	+	-	-
Groundnut	+	-	+	-	-
Melon	+	-	+	-	-
Wheat	+	-	+	-	-
Cowpea (beans)	+	-	+	-	-
Rice	+	-	+	-	-

Keys: (+) = present; (-) = absent

3.2 Amplification of ITS Region of the Fungi DNA

The ITS amplicons of all the fungal DNA isolates collected from the food samples were subjected to agararose gel electrophoresis, the DNA fragment size obtained was approximately 600bp to 700bp (Fig. 3).

3.3 Amplification of Aflatoxin Regulatory Gene of Fungi DNA

The *aflR1* gene was amplified in the DNA isolates obtained from the food samples, the resulting amplicons were used as templates for the nested PCR amplification using *aflR2* primer sets. The result obtained showed gene product of approximately 400bp (Fig. 4).

4. DISCUSSION

In this study the molecular characterization of two isolated fungal species (*A. flavus* and *A.niger*) from food sample were assessed. These fungal strains have previously been reported as contaminants of all the food samples used in this study [15,18,19, 20].

From the two fungal isolates identified from the food samples, *A. flavus* showed a higher mode of occurrence in all the food samples in all the markets. Similar report have shown that *A. flavus* is usually found abundant in food samples [17,21,22].

The *aflR* gene regulates aflatoxin biosynthesis by controlling the expression of the nor-1 and ver-1 genes [23]. In this study, nested PCR gives a positive reaction by generating the expected amplicon size of 400 bp for *aflR* gene. This result is in agreement with previous reports [15,24].

The choice of using *aflR* gene for the detection of aflatoxigenic fungi as opposed to other aflatoxin genes is due to the fact that the deletion of *aflR* in *Aspergillus* spp. has been shown to abolish the expression of other aflatoxin pathway genes [25].

These results demonstrate that *AflR* is specifically involved in the regulation of aflatoxin biosynthesis, and thus, an ideal target gene for the detection of aflatoxigenic fungi [25].

The amplification of ITS gene of the fungi isolates showed approximately DNA fragment of approximately 700bp. Further work on ITS sequence determination for proper identification of the isolates is recommended.

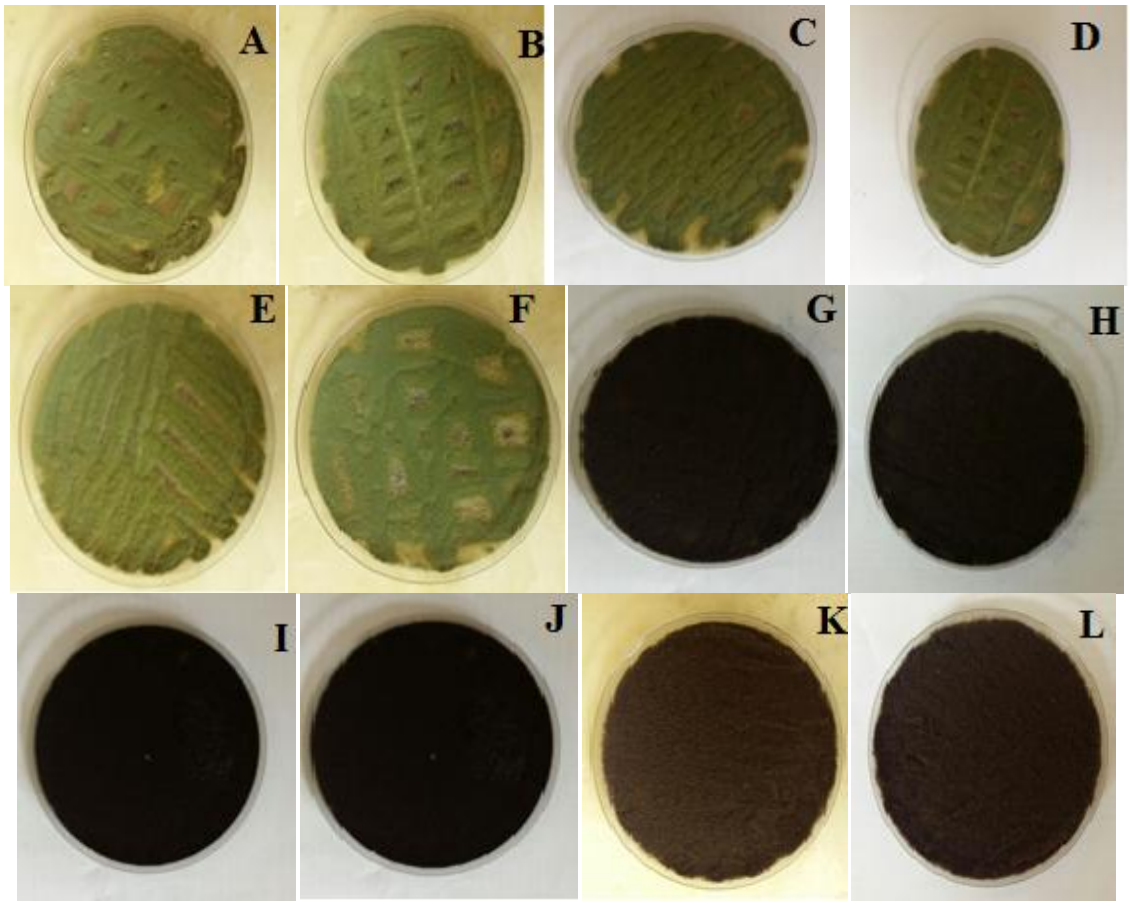

Fig. 2. Macroscopic morphology of fungal isolates obtained from mixed food samples cultured on Potato Dextrose Agar plate after 72 hours

KEYS: A- A. flavus (Rice), B- A. flavus (Wheat), C- A. flavus (Cowpea), D- A. flavus (Maize), E- A. flavus (Groundnut), F- A. flavus (Melon), G- A. niger (Rice), H- A. niger (Wheat), I- A. niger (Cowpea), J- A. niger (Maize), K- A. niger (Groundnut), L- A. niger (Melon)

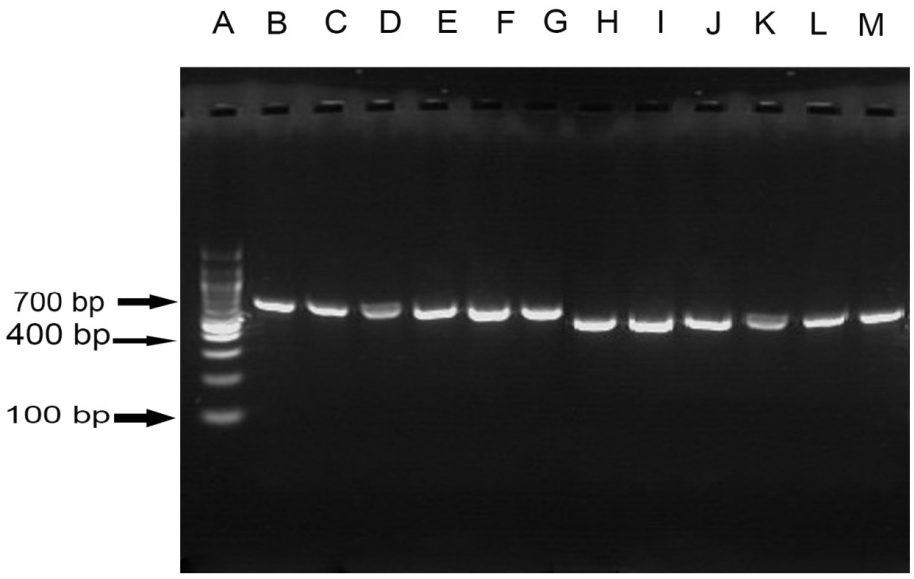

Fig. 3. PCR amplification of the Internal Transcribed Spacer (using ITS1 and ITS4 Primers) region of the DNA isolated from *A.flavus and A. niger* from all food samples

Keys: lane A- DNA ladder, B- Aspergillus flavus (Maize), C- Aspergillus flavus (Wheat), D- Aspergillus flavus (Cowpea), E- Aspergillus flavus (Rice), F- Aspergillus flavus (Groundnut), G- Aspergillus flavus(Melon), H- Aspergillus niger (Maize), I- Aspergillus niger (Wheat), J-Aspergillus niger (Cowpea), K- Aspergillus niger (Rice), L- Aspergillus niger (Groundnut), M- Aspergillus niger (Melon)

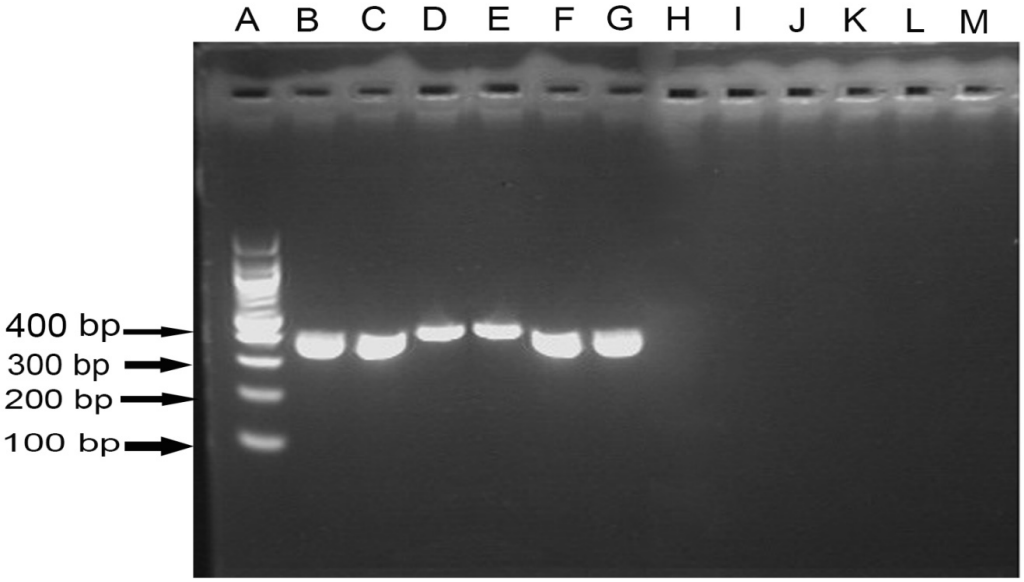

Fig. 4 PCR amplification of the *aflR* gene of the DNA isolated from *A.flavus and A. niger* using *aflR1* and *aflR2* Primers

Keys: lane A- DNA ladder, B- Aspergillus flavus (Maize), C- Aspergillus flavus (Wheat), D- Aspergillus flavus (Cowpea), E- Aspergillus flavus (Rice), F- Aspergillus flavus (Groundnut), G- Aspergillus flavus(Melon), H- Aspergillus niger (Maize), I- Aspergillus niger (Wheat), J-Aspergillus niger (Cowpea), K- Aspergillus niger (Rice), L- Aspergillus niger (Groundnut), M- Aspergillus niger (Melon)

5. CONCLUSION

This study has shown that PCR amplification of *aflR* gene has high specificity for detection of aflatoxigenic fungi in food samples obtained from selected markets in Lagos State. This approach presents a rapid method, less laborious and effective method for detecting aflatoxigenic fungi in food as opposed to analytical methods of aflatoxin quantification in food samples for monitoring of food contamination.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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