Genetic and chemical methods of ascertaining aflatoxigenicity give discordant results: A Case of *Aspergillus* species from Eastern Kenya

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Abstract

Background: Aflatoxins (AFs) are poisonous compounds produced by species of fungi belonging to the genus *Aspergillus* mainly *A. flavus* and *A. parasiticus*. However, there are some members of these species that do not produce toxins and have since become of interest for use in Biological Control programs. The species that produce AFs are believed to have clusters of aflatoxin-associated genes that confer aflatoxigenicity. However, recent findings suggest that the presence of aflatoxin genes may not imply aflatoxin production capability. We therefore examined the extent to which the presence or absence of aflatoxin-associated genes (genetic) infers aflatoxigenicity using the Dichlorvos-Ammonia (chemical) method as reference.

Results: We report a dissonance between the genetic and chemical methods of ascertaining aflatoxigenicity. Fungi (n = 314) that were morphologically identified as *A. flavus* and *A. parasiticus* were isolated from maize kernels and soil from Eastern Kenya. The fungi were further characterized to confirm their identities using a PCR-sequence analysis of the internal transcribed spacer (ITS) region of rDNA which confirmed them to be *Aspergillus* species. We selected and induced 16 isolates in YES media amended with Dichlorvos to produce AFs. Seven of the sixteen isolates were DM-AM positive (aflatoxigenic) but lacked at least one of the key aflatoxin-associated genes. Even more confounding, *A. flavus* isolate (1EM2606) had all aflatoxin-associated genes but was non-aflatoxigenic (DV-AM negative) while *A. flavus* (1EM1901) having all genes was aflatoxigenic (DV-AM positive). The genetic abundance of the AF-asscociated genes among the isolates was as follows: *aflQ* (100%), *aflD* (62.5%) followed by *aflR* (37.5%) and *aflP* (25%).

Conclusion: We report that either method alone is limited in ascertaining aflatoxigenicity among *Aspergillus* section *Flavi*. The discordance between presence of aflatoxin-associated genes and aflatoxigenicity may be due to epigenetic factors that affect secondary metabolism, or even other signaling processes that alter toxin production that still remain poorly understood.

Background

Aspergillus species of the section *Flavi* are associated with production of aflatoxins thereby contaminating food material both in field and storage conditions. The congeneric species *A. flavus* and *A. parasiticus* are principal producers of aflatoxins [1] although to a lesser extent, other fungi within the genus may produce intermediates of aflatoxin synthesis which are equally harmful [2]. Consequently, information on *Aspergillus* species diversity [3], their capacity to produce aflatoxins and the type of toxins they produce [4] is critical in risk mitigation. The production of aflatoxins was first thought to be species-specific as some species produced the toxins (aflatoxigenic) while others did not (non-aflatoxigenic) [5]. However, there are recent findings suggesting variations in capacity to produce toxins even within *Aspergillus* species [6, 7]. For this reason, it is imperative to correctly identify *Aspergillus* species and characterize their aflatoxigenic potential prior to development of viable biological control products intended to reduce mycotoxin contamination of crops in the field [5, 8].

Currently, researchers rely on diverse morphological, chemical and molecular techniques to segregate between aflatoxin producing and non-aflatoxin producing *Aspergillus* species in the section *Flavi* [3]. The choice of technique is largely determined by resource considerations mainly technical expertise, facilities and infrastructure [4]. The advancement of technology for identification of AF associated genes has greatly improved the accuracy with which species of *Aspergillus* have been classified as either aflatoxigenic or non-aflatoxigenic. However, genetic classification of aflatoxigenic potential of fungi remains a difficult task to accomplish [9] because at times results may show no significant correlation [4]. There are as many as 25 AF-genes in the cluster linked to aflatoxin production including: *aflQ*, *aflD*, *aflP*, *aflS*, *aflM*, and *aflR,* [4, 10] and addition to the list continues. Of interest currently is to find genes or sets of genes whether *aflP* and *aflQ* [9] or *aflD* and *aflS* [11] that are sufficiently predictive of aflatoxigenic potential of fungi. This will greatly simplify the process of molecular assignment of toxigenicity for *Aspergillus* species [11]. However, recent analyses of genetic and metabolic profiles of *Aspergillus* isolates and their potential to produce AFs is still confounding and inconclusive within species tested [4, 9, 10, 12].

In the context of AF production among species of *Aspergillus* section *Flavi*, workers have advanced different hypotheses to explain the variations in their findings. The observed differences are thought to be occasioned on one hand, by epigenetic factors that alter developmental changes necessary for fungi to switch from normal vegetative growth (primary metabolism) to AF production (secondary metabolism) [13]. On the other hand, the location of the AF- gene cluster on the chromosome is thought to make it inaccessible to transcription factors hence, lack of a trigger for AF production [14]. Others postulate that AF production is due to existence of other genes, yet to be characterized, that could have a bearing on AF production [15-17]; or even presence of other poorly understood signaling processes that alter toxin production [13].

In general, the existing practice is to tag *Aspergillus* isolates as toxigenic or not, based on positive identification of AF- associated genes in the fungal genome or a positive chemical test conducted on actively growing fungi. The aim of the current study was to ascertain aflatoxigenicity by combining the two (PCR-genetic and chemical) methods. Data was generated using a set of four commonly cited aflatoxin-associated genes: *aflD* (nor-1), *aflQ* (ordA)*, aflP* (omtA) and *aflR* (regulatory) and the recently developed Dichlorvos-Ammonia (DV-AM) methods for testing aflatoxigenicity. The implication of the findings is further discussed

Results

Chemical test for aflatoxigenicity

The chemical test showed that 56.25% (9 of 16) fungal isolates growing on DV-amended plates were aflatoxigenic producing a red coloration (positive test) following ammonia (AM) treatment. These were 8 *A. flavus* isolates (1EM1901, 2EM0502, 1EM4503, 2EM0602, 1EM4501, 2EM6103, 2EM3506 and 1EM4502) and 1 *A. parasiticus* (1EM0402) (Table 1 and Figure 1). Image analysis (*Image J* software)

revealed the color change occurred at the center of the plates for some isolates (Figure 1 B, C) while in others it occurred diffusely all over the entire plate (Figure 1 E, F). In contrast, the remaining 7 culture plates that had no coloration were non-aflatoxigenic (negative test).

Table 1 Results of the Dichlorvos-Ammonia (DV-AM) method and genetic analysis

		Genes			
	DV-AM Method	*aflD* (*nor-1*)	*aflQ* (*ordA*)	*aflP* (*omtA*)	*aflR* (regulatory)
1EM1901 (*A. flavus*)	+	+	+	+	+
2EM0402 (*A. parasiticus*)	+	+	+	-	-
1EM1201 (*A. flavus*)	-	+	+	-	-
1EM2901 (*A. flavus*)	-	-	+	-	+
2EM0601 (*A. parasiticus*)	-	+	+	-	+
2EM0502 (*A. flavus*)	+	+	+	-	+
2EM3503 (*A. flavus*)	-	-	+	-	-
2EM0501 (*A. flavus*)	-	+	+	-	-
1EM4503 (*A. flavus*)	+	+	+	-	-
2EM0602 (*A. flavus*)	+	+	+	+	+
2EM1201 (*A. flavus*)	-	+	+	-	+
1EM2606 (*A. flavus*)	-	+	+	+	+
1EM4501 (*A. flavus*)	+	-	+	-	+
2EM6103 (*A. flavus*)	+	-	+	-	-
2EM3506 (*A. flavus*)	+	-	+	+	+
1EM4502 (*A. flavus*)	+	-	+	-	+

+ and – denotes the presence (positive) and absence (negative) respectively.

Genetic analysis of aflatoxin gene clusters

The genetic analysis showed that most of isolates had the aflatoxin-associated genes in their genome. The abundance of the aflatoxin-associated genes was as follows: *aflQ* (*ordA*) (100%), *aflD* (*nor-1*) (62.5%), *aflR* (regulatory) (37.5%) and *aflP* (*omtA*) (25%) (Table 1). There were variations in the genes

among the species. Figure 2 is representative of gel image with the resolved bands of the respective aflatoxin-associated genes. *aflD* (*nor-1*), *aflQ* (*ordA*), *aflP*(*omtA*) and *aflR* genes were approximately 400bp, 750 bp, 1000bp and 1200bp respectively. The biological functions of the selected aflatoxin-gene cluster are provided in Table 2.

Table 2 Selected aflatoxin-associated genes and their function

Genes	Frequency	Enzyme encoded	Function
aflQ (*ordA*)	(100%),	Encodes oxidoreductase	Converts *O*-methylsterigmatocystin (OMST) and dihydro-*O*-methylsterigmatocystin (DHOMST) to aflatoxins (B1, B2, G1, and G2) [18].
aflD (*nor-1*)	(62.5%),	Norsolorinic acid reductase	Converts norsolorinic acid to averantin [19].
aflP (*omtA*)	(37.5%)	*O*-methyltransferase	Conversion of sterigmatocystin (ST) to *O*-methylsterigmatocystin (OMST) and dihydrosterigmatocystin (DHST) to dihydro-*O*-methylsterigmatocystin (DHOMST) [20].
aflR (Regulatory)	(25%)	Regulatory gene	Activates aflatoxin transcription [21]

By assessing the results of the genetic and chemical method independently, it was possible to assign aflatoxigenicity or not, to the *Aspergillus* isolates. However, when the outputs of both methods were compared, the results were confounding. There was no consistent pattern to predict aflatoxigenicity from the genetic data. While the *aflQ*-ordA gene was present in all samples, it was not sufficient to predict aflatoxigenicity. In addition, the *A. flavus* isolate 1EM1901 had all the four genes and was toxigenic in both tests. By contrast, another *A. flavus* isolate 1EM2606 also had the four genes, but was non-aflatoxigenic in the DV-AM test. This dissonance was not only among *A. flavus* but also *A. parasiticus.* The *A. parasiticus* isolate 2EM0402 which had only two of the four genes of interest was toxigenic, while the conspecific *A. parasiticus* 2EM0601 with three of the four genes was non-aflatoxigenic.

Discussion

The ubiquitous soil borne fungi in the genus *Aspergillus* are free living but become of concern when they contaminate crops and produce aflatoxins presenting serious hazard to human and animal health and negatively affect trade. The premise of Biological Control (BC) of pre-harvest aflatoxin contamination by non-aflatoxigenic strains of *A. flavus* is that there will be competition that suppresses the fungus' ability to infect or produce aflatoxin [24]. A key element for the success of BC programs using *Aspergillus* species is correct identification of candidate fungal species and accurate prediction of their aflatoxigenicity and the nature of their interaction with other species.

In the current study, the genetic and chemical methods of ascertaining aflatoxigenicity gave divergent results implying that the selected AF-genes could not predict (non-)aflatoxigenicity among tested *Aspergillus* species. Similarly, a negative DV-AM test did not predict presence or absence of AF-associated genes. This incongruence between the presence of AF- genes and aflatoxin production could not be explained. The DV-AM method has been reported successful in segregating aflatoxigenic and non-aflatoxigenic strains of *A. flavus* from Mexico [25]. The findings of the current study, to the best of our knowledge, demonstrate confounding results derived from an effort to predict aflatoxigenicity among *Aspergillus* species isolated from Eastern Kenya-a known aflatoxicosis hotspot.

Earlier work had proposed *aflQ* (*ordA*) and *aflD* (*nor-1*) to be ideal markers of aflatoxigenic potential [12, 23, 26]. On the contrary, our findings indicate the above genes are dominant among *Aspergillus* species but insufficient to assign (non-)aflatoxigenicity. This corroborates the work of Okoth, et al. [4] suggesting absence of correlation between the presence of AF-associated genes and the production of aflatoxins. An attempt to explain these variations may require in-depth investigation into the interaction of metabolic pathways that could interact with the AF biosynthetic pathway. This was not the scope of the current work. According to Price, et al. [21], the gene *aflR* could regulate other genes outside the AF biosynthetic cluster driving AF production. The proximity of gene clusters is believed to influence the expression of adjacent genes. Price, et al. [21] linked the presence of the genes: *hypB, nadA, hlyC,* and *niiA* to the regulatory effect of *aflR*. In the current study, it may be difficult to assign an overarching role in aflatoxin production to the *aflR* regulatory gene. This is because some non-aflatoxigenic isolates had the *aflR* regulatory gene while other toxigenic that did not have it.

It is possible that other factors such as mutations in the AF-associated genes could affect aflatoxin production among species within section *Flavi* [12, 27]. The existence of inter- and intra-specific mutations within the genes of interest likely affect target binding sites of the primers used [10, 28]. However, that requires further investigation. We are inclined to concur with Okoth, et al. [4] and Oloo, et al. [11] that there is need to combine several methods such as: Chemical, Molecular (using more genes) and Metabolic profiling to establish aflatoxigenic potentials of fungi prior to their use as candidates for Biological Control programs.

Conclusions

It appears that the current preferred methods for ascertaining aflatoxigenicity do not converge sufficiently, due to unknown reasons. Resolution of this stalemate ought to have great implications on Biological control programs using non-aflatoxigenic *Aspergillus* species.

Methods

Identification and culturing of fungi

Aspergillus species used in this study were isolated from maize kernels collected in Eastern Kenya aflatoxicosis hot-spot [29]. The fungi were sub-cultured following standard protocols then identified to species level following morphological and colony characteristics using *Aspergillus* specific keys [30]. The selection of the fungi in this study was based on their isolation frequency from the maize samples. This gave a total of 16 most frequently isolated *Aspergillus* species. Molecular identification of the fungi was carried out using the Internal Transcribed Spacer (ITS1–5.8S-ITS2 cluster) regions of the ribosomal DNA gene cluster [4]. The fungi were then sub-cultured on Czapek Dox Agar medium (HiMedia Laboratories Pvt. Ltd), then incubated at 28°C for five days. The fungi were coded depending on the season and area of collection and maize sample number*: A. flavus* were coded as: [1EM1901, 1EM1201, 1EM2606, 1EM2901, 1EM4501, 1EM4501, 1EM4503, 2EM0501, 2EM0502, 2EM0602, 2EM1201, 2EM3503, 2EM3506, and 2EM6103] and *A. parasiticus* coded as: [2EM0402 and 2EM0601].

Dichlorvos-ammonia (DV-AM) test for aflatoxigenicity

For the DV-AM method, the fully grown isolates initially cultured on the Czapek Dox Agar medium were sub-cultured on aflatoxin-inducing Yeast Extract Sucrose (YES) medium (amended with 50mg/L streptomycin sulfate and penicillin, Zhonghuo Pharmaceuticals, China) and maintained for seven days at 28°C. The culturing and testing for aflatoxigenicity of the isolates on the aflatoxin-inducing YES media was sequentially done following the protocols for the DV-AM method as described by [25]. The method briefly involved: (i) Dichlorvos diluted with methanol in 250 fold ratio (Amiran Kenya Ltd) was premixed with the media prior to solidification, (ii) the isolates were incubated in darkness at 28°C for 5 days, (iii) 0.2 mL of ammonium hydroxide solution was poured onto the inside of the lid of the petri dish plate used to set up the DV-AM method after the 5-day incubation period, (iv) the isolates were categorized as either aflatoxigenic or non-aflatoxigenic depending on their ability to produce aflatoxins (shown by a characteristic red coloration at the underside of the plates), and (v) the plate images of the aflatoxigenic isolates were further analyzed with ImageJ 1.x software for enhanced detection (Figure 1 C, F).

Identification of aflatoxin-associated gene clusters

The fungal isolates used for molecular identification were grown in Potato Dextrose Agar medium (PDA; pH 6.0) and maintained at 28 °C for five days. Afterwards, the periphery of the exponentially growing fungal mycelium (50-100 mg) was excised aseptically using a sterile scalpel. DNA extraction was done using Quick-DNA™ Fungal/Bacterial Kit (Zymo Research). Finally, 100 µL of DNA was eluted for amplification. Four aflatoxin-specific primers (Inqaba Biotechnical Industries (Pty) Ltd (South Africa)) were used to amplify genes (*aflD, aflQ,* and *aflP*) encoding for enzymes involved in the aflatoxin biosynthesis and regulation (*aflR*) of the pathway (Table 3). The four genes selected for this study were chosen based on their direct role in the final production of aflatoxins as reported in the literature (Table 2).

Table 3 Aflatoxin-associated primer-pair combinations for *Aspergillus* species

Primer code	Target gene	Primer Sequences	References	T_m	Purification	Modifications
AflD-1for	*aflD* (*nor-1*)	Nor1-F (5′-ACC GCT ACG CCG GCA CTC TCG GCA C-3′)	[22]	74.42	Standard	None
AflD-2rev		Nor1-R (5′-GTT GGC CGC CAG CTT CGA CAC TCC G-3′)		72.78	Standard	None
AflQ-1for	*aflQ* (*ordA*)	Ord1-gF (5′-TTA AGG CAG CGG AAT ACA AG-3′)	[23]	58.35	Standard	None
AflQ-2rev		Ord1-gR (5′-GAC GCC CAA AGC CGA ACA CAA A-3′)		64.54	Standard	None
AflP-1for	*aflP* (*omtA*)	AflP-1for (5′-AGC CCC GAA GAC CAT AAA C-3′)	[9]	60.16	Standard	None
AflP-2rev		AflP-2rev (5′-CCG AAT GTC ATG CTC CAT C-3′)		60.16	Standard	None
AflR-1for	*aflR*	AflR-1for aflR (5′-AAG CTC CGG GAT AGC TGT A-3′)	[9]	60.16	Standard	None
AflR-2rev		AflR-2rev (5′-AGG CCA CTA AAC CCG AGT A-3′)		60.16	Standard	None

During the reaction, a total reaction volume of 25 µL consisting of 12.5 µL of One Taq® 2X Master Mix (New England Biolabs), 0.2 µL of DNA template (< 1000 ng), 0.5 µL (0.2 µM) of each (forward and reverse) of the primers and 9.5 µL of nuclease-free water. The mixture was gently mixed by priming pipettor at least 4 times and spun prior to PCR. PCR tubes were transferred to a preheated thermocycler at 94 °C for 3 minutes followed 30 cycles consisting of 1 minute of denaturation at 94 °C, 1 minute of annealing at 57 °C and 1 minute of extension at 72 °C. A final extension of 10 minutes at 72 °C and a storage temperature of 4 °C were considered. The PCR products were then subjected to gel electrophoresis using 1 % agarose gel pre-cast with Ethidium Bromide. The optimized conditions for the process involved a constant supply of voltage (50 V (41mA)) for 75 minutes in a Tris-borate-EDTA (TBE) buffer electrophoretic chamber. The gel was visualized using Automatic Gel Imaging System (Peiqing Science and Technology Co., Ltd).

Statistical analysis

All experiments were replicated thrice and data is presented in percentages for the parameters analyzed. *ImageJ* 1.x software was used for color enhancements in plates with fungal colonies.

List Of Abbreviations

AF(s): Aflatoxin(s)

DHOMST: Dihydro-O-methylsterigmatocystin

DV-AM: Dichlorvos-Ammonia

OMST: O-methylsterigmatocystin

ST: Sterigmatocystin

TBE: tris-borate-EDTA

YES: Yeast Extract Sucrose

ITS: Internal Transcribed Spacer

Declarations

Acknowledgements

Not applicable.

Authors' contributions

LKM: Performed the experiments, generated data and drafted the manuscript; MAO and FJT: Designed the research topic, sourced funding, supervised the project and reviewed the manuscript; HMM: Provided fungal molecular expertise, supervised the project, analyzed the data and reviewed the manuscript. All authors approved the final draft.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

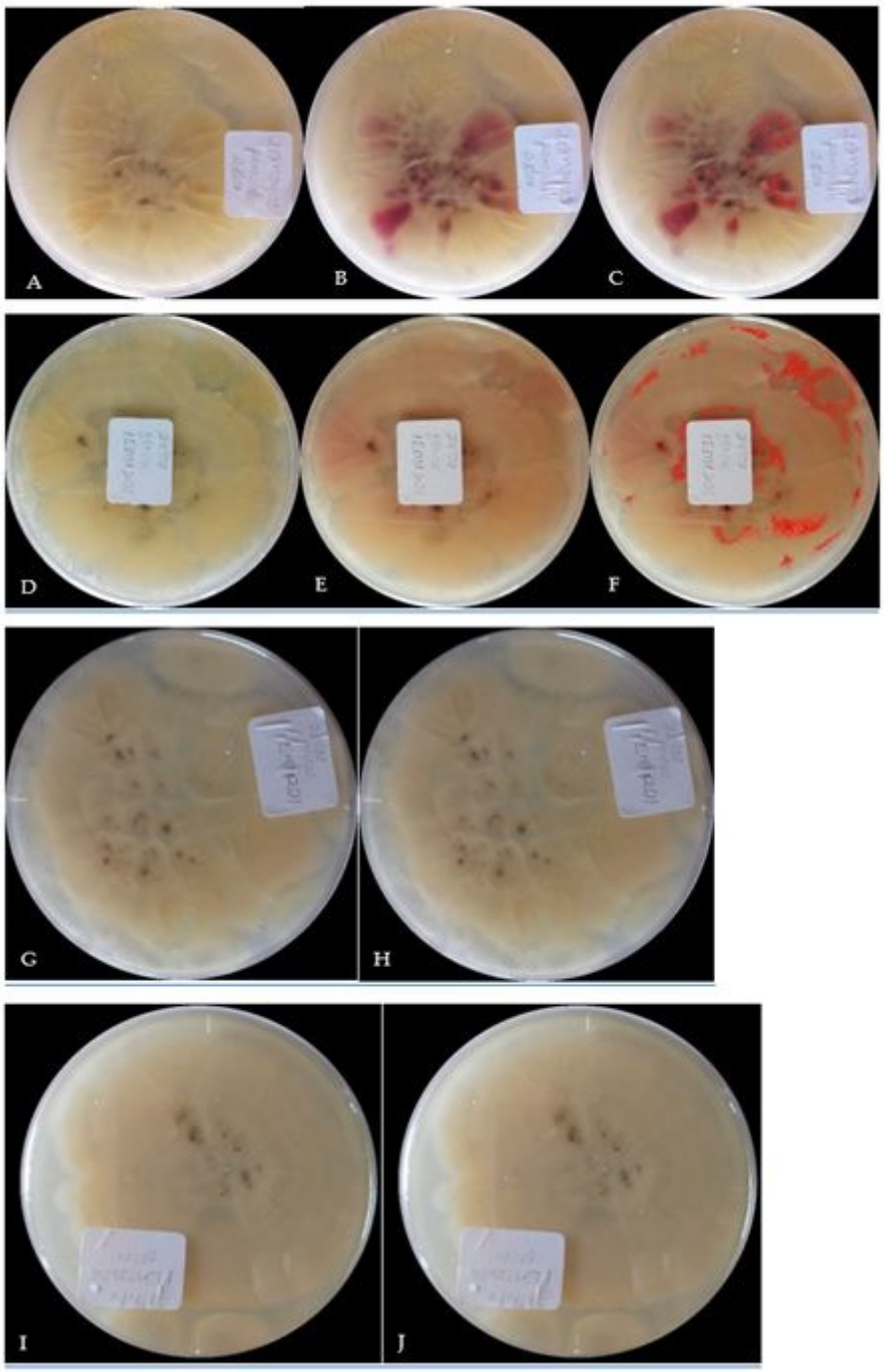

Figure 1

Dichlorvos-ammonia (DV-AM) method for aflatoxin detection in YES aflatoxin inducing media; (A) and (D) Fungal plates treated with Dichlorvos (DV) before ammonia (AM) treatment, (B) and (E) after ammonia treatment and (C) and (F) after ImageJ 1.x processing for isolates 2EM0402 and 1EM1901 respectively; (G) and (I) Fungal plates treated with DV before AM treatment and (H) and (J) after AM treatment for isolates 1EM1201 and 1EM2606 respectively.

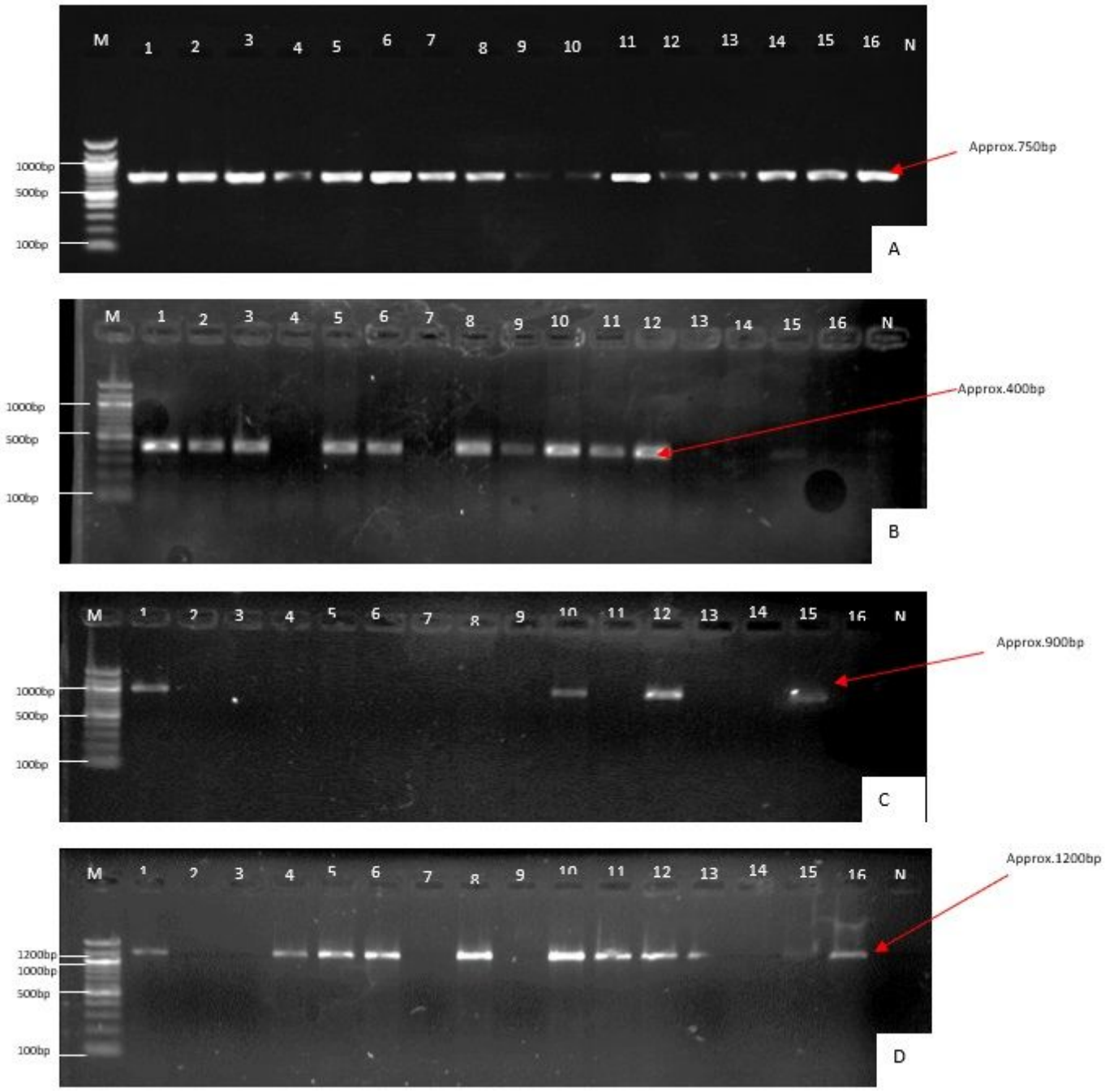

Figure 2

PCR products expressing the presence of aflQ (A), aflD (B), aflP (C) and AflR (D) genes across the isolates. M- Molecular DNA 100 bp ladder (New England Biolabs); 1-1EM1901 (positive control) (A. flavus); 2- 2EM0402 (A. parasiticus); 3-1EM1201 (A. flavus); 4-1EM2901 (A. flavus); 5- 2EM0601 (A. parasiticus); 6-2EM0502 (A. flavus); 7-2EM3503 (A. flavus); 8-2EM0501 (A. flavus); 9-1EM4503 (A. flavus); 10-2EM0602 (A. flavus); 11-2EM1201 (A. flavus); 12-1EM2606 (A. flavus); 13- 1EM4501 (A. flavus); 14- 2EM6103 (A. flavus); 15-2EM3506 (A. flavus); 16-1EM4502 (A. flavus); N- Negative control