# Development and Optimization of a PCR–RFLP Method for Differentiation of *Aspergillus flavus* from Aspergillus *parasiticus*

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## ABSTRACT:

Aflatoxins are highly toxic secondary metabolites produced by several *Aspergillus* species, pose a global threat to food and feed safety, making rapid and reliable identification of aflatoxigenic species essential for effective surveillance and risk mitigation. Here, we report the development and validation a simple PCR–RFLP assay that discriminates between the closely related species *Aspergillus flavus* and *Aspergillus parasiticus* by targeting conserved regions of the aflatoxin biosynthetic pathway for use in feed surveillance.

A total of 42 fungal isolates from feed samples were identified as *Aspergillus* species and genomic DNA was extracted from the cultured mycelial biomass for further molecular analyses. The conserved primer sets to amplify three genes related to aflatoxin production pathway: *aflD* (nor-1), *aflR*, and *aflP* (omtA). The PCR products were digested with the restriction enzymes XbaI and XhoI and resolved them using 2% agarose gel. The RFLP patterns compared with the predicted digestion patterns from reference genomes to check their agreement and ability to provide accurate diagnosis.

Distinct RFLP patterns were obtained for *A. flavus* whereby for each of the three amplicons there were two diagnostic fragments (e.g. 321/139 bp for *aflD*). In contrast, the PCR products for *A. parasiticus* did not produce the same restriction sites, and no digestion was seen in this species. The assay consistently discriminated *A. flavus* from *A. parasiticus*.

The recommended PCR–RFLP assay can be a rapid and cost-effective technique for identifying *A. flavus* from *A. parasiticus* in feed samples. However, using only this enzyme selection does not discriminate all clinically or agriculturally pertinent genera of *Aspergillus*. We recommend using this assay with added and new restriction enzymes, or coupling with other molecular analysis in order to allow for complete species discrimination. Ultimately, the performance should be validated with a variety of field isolates before using it as a standard diagnostic method.

**Keywords:** *Aspergillus flavus, Aspergillus parasiticus*, PCR-RFLP, *aflD*, *aflR*, *aflP*

## 1. INTRODUCTION:

The *Aspergillus* genus includes filamentous fungi that infect various substrates such as soil, water, and organic debris and easily spread through airborne conidia (1). *Aspergillus* species frequently contaminate agricultural produce and elaborate a wide range of mycotoxins (2, 3). The most toxic among these are aflatoxins with mutagenic, hepatotoxic, and carcinogenic activities. Due to their effects on human health, the International Agency for Research on Cancer categorizes aflatoxins as Group 1 carcinogens (4, 5).

Two of the species, *A. flavus* and *A. parasiticus*, are largely accountable for the widespread prevalence of aflatoxin contamination of agricultural commodities and animal feeds. Under suboptimal storage and handling conditions, especially high temperature and humidity, these fungi can proliferate and aflatoxins may be produced, infiltrating the food chain through both direct and indirect pathways (6, 7). For example, lactating animals that ingest contaminated feed are capable of excreting aflatoxin $M_1$ in milk, leading to subsequent dairy product contamination and posing significant risks of hepatotoxicity, immunosuppression, and even acute toxicity in humans (8, 9).

Aflatoxin biosynthesis is a highly regulated process comprising at least 18 enzymatic steps that convert acetyl-CoA to the four major aflatoxins ($B_1$, $B_2$, $G_1$, $G_2$). Primary genes in this pathway, including *aflD* (nor-1), *aflR*, and *aflP* (omtA), are clustered in a 75 kb gene cluster on chromosome III of the Aspergillus genome (10, 11). While *A. flavus* produces mainly the B-group aflatoxins, *A. parasiticus* produces both B- and G-group toxins, indicative of species-specific pathway regulation and enzyme specificity differences (12, 13).

Accurate identification of these closely related species is essential for effective aflatoxin surveillance, yet conventional morphological and biochemical assays lack the resolution to distinguish minor genetic variations, particularly in non-coding regions. Molecular techniques such as species-specific PCR, qPCR, and multi-locus sequencing have improved detection sensitivity but often require multiple assays or specialized equipment (14, 15).

PCR–RFLP provides a simplified option by coupling a single PCR amplification of target loci with restriction fragment length polymorphism analysis. After digestion with one or more restriction endonucleases, amplified DNA fragments are separated by electrophoresis, producing species-specific banding patterns. This is a robust and time-saving method that has been used successfully in fungal diagnostics in earlier work (16–18).

To address the need for quick and affordable detection of aflatoxin-producing *Aspergillus* species in cattle feed, we developed a PCR-RFLP assay aimed at distinguishing *Aspergillus flavus* from *Aspergillus parasiticus*. The assay focuses on three important genes in the aflatoxin biosynthetic pathway (*aflD*, *aflR*, and *aflP*) and uses the restriction endonucleases XbaI and XhoI.

## 2. MATERIALS AND METHODS:

### 2.1. Fungal Isolates and Culture Conditions

Based on the previous study (19), a total of 42 isolates from feed samples were chosen for further analysis. These isolates were found to be *Aspergillus spp.* based on Based on morphological and molecular characteristics. The isolates were cultured on Czapep Yeast Extract Agar (CYA) and Malt Extract Agar (MEA) media at 25°C and 37°C for seven days. The daily evaluation taken include the size, texture, pigmentation (surface and reverse), and the production of exudate. Microscopic observations were conducted using bright-field and phase-contrast microscopy (Nikon Eclipse E200) from lactophenol cotton blue-stained mounts. Diagnostic features including conidiophore structure, vesicle shape, phialide arrangement, conidial head type, and ornamentation were carefully documented. Species identification followed established taxonomic keys and reference descriptions (19).

### 2.2. Genomic DNA Extraction

Conidial suspensions (1 mL) were cultured in 50 mL of Sabouraud Dextrose Broth at 30 °C with shaking (150 rpm) for 72 h obtaining mycelial biomass. The mycelia were collected by vacuum filtration, washed twice with ice-cold PBS (pH 7.4), flash frozen in liquid nitrogen, and lyophilized for 24 h. Approximately 50 mg of freeze-dried mycelium was ground in liquid nitrogen to a fine powder. The powder was lysed in buffer (100 mM Tris–HCl pH 8.0, 50 mM EDTA, 2% SDS) with 0.5 mm acid-washed glass beads and vortexed; phenol:chloroform:isoamyl alcohol (25:24:1) was added in equal volume for organic extraction. Following centrifugation at 12,000 × g for 10 min at 4 °C, the aqueous phase was removed, and the DNA precipitated in isopropanol for 1 hour at −20 °C. Pellets were washed twice with 70% ethanol, air dried while still in the tubes, and resuspended in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). The quantity and purity of the DNA were assessed using a NanoDrop 2000 spectrophotometer and DNA integrity was visualized on 0.8% agarose gels.

### 2.3.Primer Design and PCR Amplification

Gene-specific primers for *aflD* (nor-1), *aflR* and *aflP* (omtA) were designed using Oligo 7 software using reference sequences from GenBank (Table 1). Each 50 µL PCR contained 150 ng of genomic DNA, 5 µL of 10 × PCR Buffer (100 mM Tris–HCl, 500 mM KCl, pH 8.3), 1.5 mM $MgCl_2$, 200 µM of each dNTP (Thermo Fisher Scientific), 0.3 µM of each primer and Taq DNA polymerase

(2.5 U, RepliQa® HiFi, Quantabio). Each amplification cycle was performed on an Eppendorf Mastercycler Pro beginning with three minutes of initial denaturation at 95 °C, then followed by 35 cycles of 95 °C for 30 seconds, annealing at the primer specific temperature for 30 seconds, and extending the reaction at 72 °C for 45 seconds, concluding with five minute of final extension at 72 °C. PCR products were visualized on 1.5 % agarose gels stained with GelRed® (Biotium) and cleaned up using the QIAquick PCR Purification Kit (Qiagen).

**Table 1.** Nucleotide sequences of primers targeting the *aflD*, *aflP*, and *aflR* genes used in this

| Gene name | Sequence | Gene length |
|---|---|---|
| *afl*D-F | 5′-CTCATCACACGCAGGCATCGG-3′ | 702 |
| *aflD*-R | 5′-AGATGCCTGCCACACTGTCT-3′ | |
| *aflP*-F | 5′-CCCATCTCGATAGCGCCTG-3′ | 611 |
| *aflP*-R | 5′-GCCACCCATACCTAGATCAAAGC-3′ | |
| *aflR*–F | 5′- AGAGCTACTGAACGTCCCAT-3′ | 1458 |
| *aflR*–R | 5′-ATCAGGTTGCACGAACTGTCC-3′ | |

### 2.4.Restriction Fragment Length Polymorphism (RFLP) Analysis

The XbaI and XhoI were selected based on analysis of conserved and variable regions in the *aflP*, *aflR*, and *aflD* genes. Purified amplicons (12 µL) were digested in 15 µL reactions that included 5 U of a restriction enzyme (XbaI or XhoI) (New England Biolabs) and 1.5 µL of the corresponding 10× NEBuffer. Digests were incubated at 37 °C for one hour, then inactivated at 65 °C for 20 minutes. Subsequently, ten microliters of each digest were combined with loading dye and electrophoresed on 2 % agarose gels in 1× TAE buffer at 100 V for 90 minutes. After staining with 0.5 µg/mL of ethidium bromide, gels were imaged on a Gel Doc 2000 system (Bio-Rad). Fragment sizes were estimated using a 100 bp DNA ladder (Thermo Fisher Scientific).

### 2.5. Controls and Reproducibility

Validation of assay was performed by genomic DNA from *Aspergillus flavus* (ATCC 204304) and *Aspergillus parasiticus* (ATCC 15517) as positive controls. Negative controls included no-template and no-enzyme reactions to check for contamination and nonspecific enzyme activity. All

PCR and RFLP experiments were done in duplicate on different days. This approach assessed reproducibility and ensured consistent banding patterns for reliable species identification.

## 3. RESULTS:

The initial species identification was performed by using the available morphological keys. According to the observable phenotypic similarities among species of the *Aspergillus flavus* species complex (includes species such as *A. flavus*, *A. oryzae*, and *A. parasiticus*) a tentative species identification was made and further confirmed by molecular analysis. The species were identified as *A. flavus* (13 isolates, 30.9%), *A. oryzae* (11 isolates, 26.2%), *A. tamarii* (7 isolates, 16.7%), *A. parasiticus* (6 isolates, 14.3%), and *A. tubingensis* (2 isolates, 4.8%). Due to insufficient diagnostic morphological features, three isolates (7.1%) were identified as *Aspergillus sp.* A summary of species identification is provided in Table 2.

**Table 2.** Morphological and molecular characterization of 42 *Aspergillus* isolates from feedstuff, including species identity, number of isolates, and sampling regions.

| ***Aspergillus* species** | **Number/ Percentage** | **Isolation source** | **Aflatoxin production on TLC** |
|---|---|---|---|
| *A. flavus* | 13 / 30.9 | Corn , wheat, barley | Positive |
| *A. parasiticus* | 6 / 14.3 | Barley, Soybean Meal | Positive |
| *A. tamarii* | 7 / 16.7 | Barley, wheat | Negative |
| *A. tubingensis* | 2 / 4.7 | Corn, Soybean Meal | Negative |
| *A. oryzae* | 11 / 26.2 | Barley, wheat | Negative |
| *Aspergillus sp.* | 3 / 7.1 | Corn, Barley, Soybean Meal | Negative |

The three aflatoxin genes, *aflD*, *aflP*, and *aflR*, was successfully amplified from genome of all aflatoxin-producing isolates of *Aspergillus*. The PCR products showed uniform sizes across isolates, including 702 bp for *aflD*, 1458 bp for *aflR*, and 611 bp for *aflP* (Fig. 1; Table 3). After digestion by XhoI and XbaI enzymes, the species-specific fragments were observed. In *A. flavus*, all three genes were digested and the fragment size ranged between 298-432 bp for *aflD* (using XhoI), 148-1317 bp for *aflR* (using XhoI), and 150-460 bp for *aflP* (using XbaI) (Fig. 2). In contrast, no gene fragment could be digested by either of the enzymes in *A. parasiticus* and size of the fragments also remained unaltered.

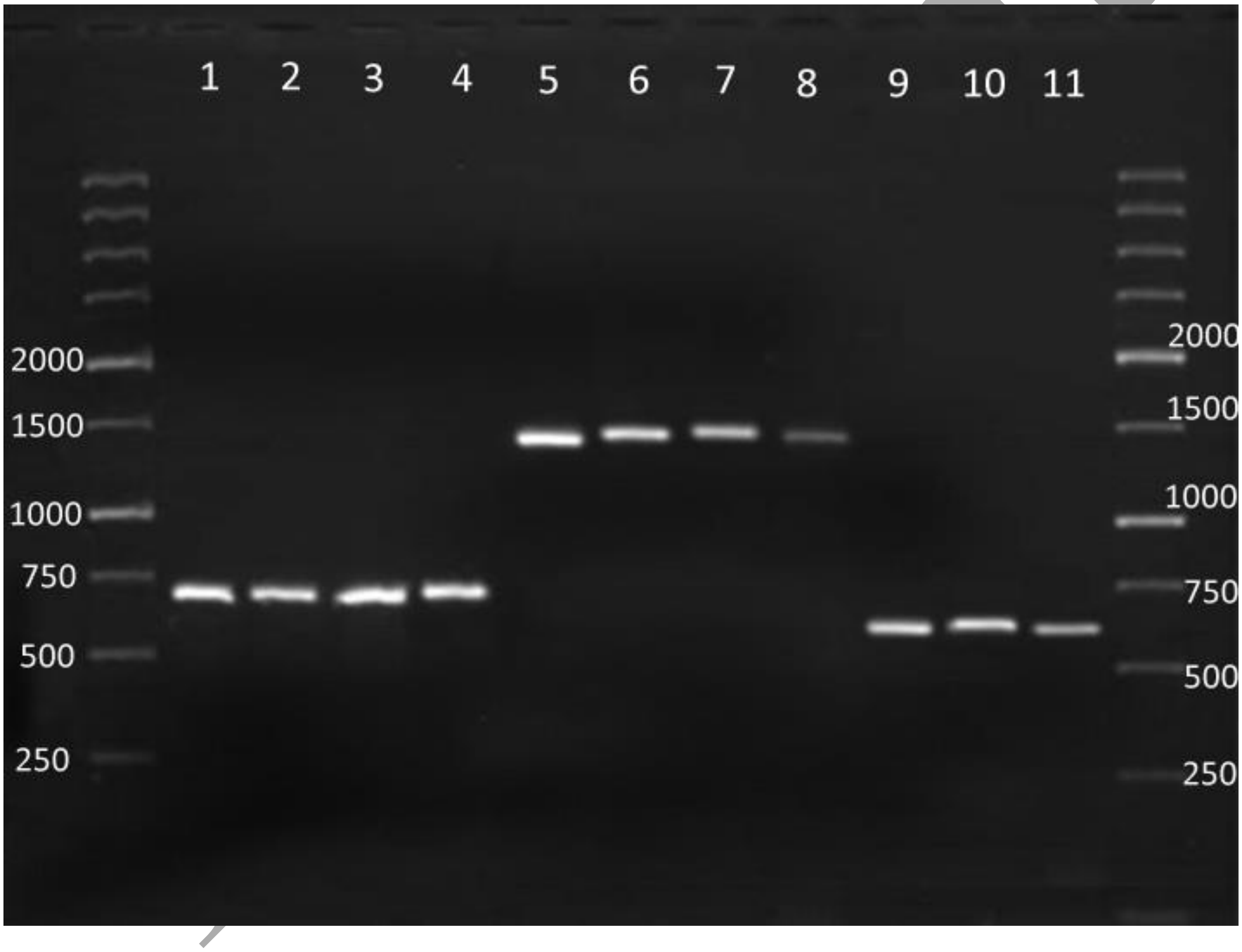

**Figure 1.** Agarose gel electrophoresis of PCR amplicons for aflatoxin biosynthesis genes in aflatoxigenic *Aspergillus* isolates. Lanes 1–4 display the ~702 bp *aflD* fragment, lanes 5–8 the ~1458 bp *aflR* fragment, and lanes 9–11 the ~611 bp *aflP* fragments. A 100 bp DNA ladder is shown in the first and last lanes for size reference.

**Table 3.** Amplicon sizes of *aflD*, *aflR*, and *aflP* genes in representative *Aspergillus* isolates before and after digestion with XhoI and XbaI restriction endonucleases.

| ***Aspergillus species*** | **PCR product size (bp)** | | | **Fragment size after digestion (bp)** | | |
|---|---|---|---|---|---|---|
| | *aflD* | *aflR* | *aflP* | *aflD* (XhoI) | *aflR* (XhoI) | *aflP* (XbaI) |
| *A. flavus* | 702 | 1458 | 611 | 298- 432 | 148-1317 | 150-460 |
| *A. parasiticus* | 702 | 1458 | 611 | Intact | Intact | Intact |

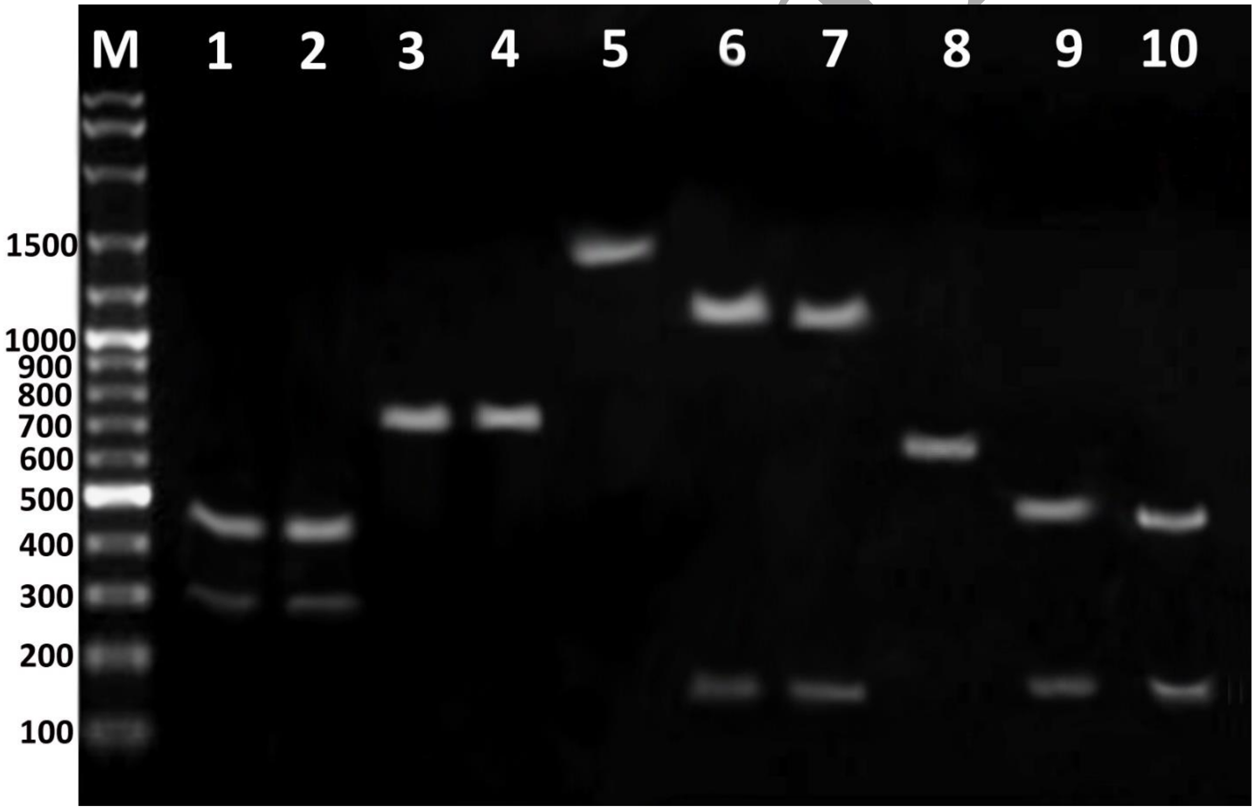

**Figure 2.** Restriction fragment length polymorphism of *aflD*, *aflR*, and *aflP* amplicons following digestion with XhoI and XbaI. In lanes 1–2, XhoI cleavage of the *aflD* product yields 432 bp and 298 bp fragments; lanes 3–4 show the corresponding undigested *aflD* control. Lane 5 presents the undigested *aflR* amplicon, whereas lanes 6–7 display XhoI-generated fragments of 1317 bp and

148 bp. Lane 8 depicts undigested *aflP*, and lanes 9–10 show XbaI digestion products of 460 bp and 150 bp. M, 100 bp DNA marker.

## 4. DISCUSSION:

Rapid and reliable detection of aflatoxigenic *Aspergillus* species continues to be a vital part of effective control strategies in both dairy and animal feed products (21). For many years, *Aspergillus* spp. have been identified by the use of visual colony morphology, resulting in a phenotypic examination of conidial structures microscopically by the laboratory or processing employee applying the testing methods. Although these phenotypic methods provide the ability to distinguish broad taxonomic groups, they require labor-intensive time, are slow and are often complicated by subtle interspecific variability within groups of species making them difficult to identify accurately (15, 22).

Genomic analyses have demonstrated that *A. flavus* and *A. parasiticus* had very similar genome sizes along with a high degree of homology between nucleotide sequences, leading to their misidentification by PCR assays that used conserved sequences (18, 23). Molecular fingerprinting strategies based on genomic polymorphism has emerged in response to the inability to separate *A. flavus* and *A. parasiticus*. Random amplified polymorphic DNA (RAPD) is one of the molecular strategies that have been used for this, but RAPD has poor reproducibility and banding patterns using low stringency annealing conditions and therefore is not appropriate for diagnostic or epidemiological purposes (24).

PCR-RFLP analysis has emerged as an effective method for differentiating closely related *Aspergillus* species (25). However, recent PCR-RFLP approaches offer more targeted and efficient species differentiation. Somashekar et al. successfully distinguished these species by targeting the aflR gene and using *PvuII* digestion (26). Similarly, El Khoury developed a PCR-RFLP method targeting the aflR-aflJ intergenic spacer and employing BglII for differentiation (27). They further described this protocol, highlighting its simplicity and cost-effectiveness compared to conventional sequencing (28).

Similarly, PCR-RFLP of the β-tubulin gene using *AlwI* enzyme has generated unique patterns for six clinically significant Aspergillus species, offering a reliable alternative to expensive DNA sequencing (29). The method's versatility has been further demonstrated by its application to the ITS1-5.8S rDNA-ITS2 region and portions of the β-tubulin and calmodulin genes, using various restriction enzymes to determine variability among Aspergillus isolates (30).

According to related studies, we designed a PCR-RFLP assay targeting three major genes involved in aflatoxin biosynthesis (*aflR*, *aflP*, and *aflD*), with XbaI and XhoI restriction endonucleases to identify specific polymorphisms for discriminating *A. flavus* from *A. parasiticus*. By digesting with these two restriction enzymes, we obtained specific patterns for *A. flavus* and *A. parasiticus* to easily distinguish both species from each other for feed samples at the species level. This is a simple approach that combines a targeted PCR with a simple restriction digest which constitutes a rapid and cost effective method to screening and differentiation of *A. flavus* from *A. parasiticus*, which are the two major toxigenic *Aspergillus* species found in cattle feeds.

However, some limitations should be considered for future studies. The restriction sites could be gained or lost by mutations, and this might result in incorrect classification of uncharacterized isolates. Moreover, only two restriction enzymes were used in the present study, and some *Aspergillus* species, having differences in aflatoxin gene sequences and possibly important in clinical or geographical contexts, might be overlooked. Therefore, we strongly recommend the use of additional restriction enzymes, and complementary approaches, such as high-resolution melting (HRM) analysis, could also be incorporated to enhance assay resolution.

## 5. CONCLUSION:

Based on these precedents, we develop a method of PCR-RFLP to evaluate three key aflatoxin biosynthesis genes, *aflR*, *aflP*, and *aflD,* and use the restriction endonucleases XbaI and XhoI to identify diagnostic polymorphisms. The information obtained from these enzymes provided unique fragment patterns for *A. flavus* and *A. parasiticus*, performing unequivocal intraspecies differentiation of feedstuff isolates. This is a simple approach that combines a targeted PCR with a simple restriction digest to achieve rapid and cost effective differentiation of the two main toxigenic species found in cattle feeds. The assay should be interpreted as a screening method rather than a definitive taxonomic test. Improving species-level resolution will require further optimization. Finally, validation on larger, geographically and genetically diverse isolate collections, including blinded comparisons to sequence-based reference methods, would be necessary before broader adoption in high-throughput diagnostic workflows aimed at reducing aflatoxin risk.

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**Authors' Contribution:**

Study concept and design: M.T and N.S, Acquisition of data: M.T , Analysis and interpretation of data: N.S, M.T, Drafting of the manuscript: N.S.

**Ethics:** All ethical standards were observed. The authors confirm no plagiarism, data fabrication, or inappropriate data manipulation.

**Conflict of Interest:** The authors do not have any conflict of interest.

No. Data Availability the data that support the findings of this study are available on request from the corresponding author.

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