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ORIGINAL ARTICLE

Aflatoxin-producing *Aspergillus flavus* from food samples in Nepal

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ABSTRACT

Background and Objectives: Maize and peanuts, staple foods in Nepal, are highly susceptible to fungal contamination, particularly with aflatoxin-producing *Aspergillus flavus*. This study aimed to detect aflatoxin-producing *A. flavus* in maize and peanut samples collected from different regions of Nepal.

Materials and Methods: A total of 80 maize and 20 peanut samples were collected randomly from markets and households. Disinfected samples were inoculated on Potato Dextrose Agar and incubated at 28°C for 48 hours. *A. flavus* was identified by conventional cultural methods. Aflatoxin production was screened on *Aspergillus* Differential Medium (ADM) and confirmed through Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC).

Results: Fungal contamination was detected in 67.0% of samples, with a significantly higher rate in household-stored samples ($p = 0.00039$). *A. flavus* was the predominant species, particularly in maize (72.5%). Of 50 *A. flavus* isolates, 15 (30%) were aflatoxin positive on ADM, with 11 (73.3%) being confirmed by TLC. HPLC revealed AFB1 as the most prevalent in both maize and peanuts, while AFB2 was restricted to maize.

Conclusion: Maize and peanuts are highly susceptible to contamination with aflatoxin producing *A. flavus* in Nepal, particularly in household-stored samples, emphasizing significant food safety concerns.

Keywords: Aflatoxin; *Aspergillus flavus*; Cereals; Food safety

INTRODUCTION

Maize, considered as a key staple food in developing countries like Nepal, is susceptible to post-harvest fungal contamination (1-3), and mycotoxin-producing *Aspergillus* species are of major concern (2). Aflatoxins, produced by *Aspergillus* species, are carcinogenic, mutagenic, teratogenic, and immunosuppressive (3-6). Globally, around 25% of food is contaminated with aflatoxins (7-13). Recent studies have also highlighted that aflatoxin contamination in staple foods represents a critical public health concern. A survey conducted in Iran reported that 98% of rice samples were contaminated, resulting in a probable daily intake that exceeds established safety thresholds (14). To mitigate these risks, biological detoxification strategies have demonstrated considerable efficacy. Specifically, the incorporation of *Saccharomyces cerevisiae* and *Lactobacillus plantarum* in bread production resulted in a complete (100%) reduction of aflatoxin B1 and ochratoxin A following the baking process (15). Similarly, in dairy products, probiotic bacteria effectively reduced aflatoxin M1, providing a safe decontamination practice (16).

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Corn, in particular from the plains of Terai to the hill areas of Nepal, is highly vulnerable to *Aspergillus* contamination leading to aflatoxin production during storage and transportation (17-21). The four major aflatoxin types, aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2), are the secondary metabolites produced by the fungus *Aspergillus* species. Regular consumption of aflatoxin-contaminated foods can lead to severe health consequences (3, 9, 13, 22). Peanuts, maize, and their products sold in the markets of developing countries of Asia and Africa are highly contaminated with aflatoxins, AFB1, AFB2, AFG1, and AFG2 at an alarming level (17, 22-25). The type and extent of aflatoxin contamination is influenced by various factors, such as the species or strain of the fungi, food type, temperature, and humidity of the environment (13, 21).

According to the International Agency for Research on Cancer, mycotoxins and aflatoxins (AFTs) are classified as Group 1 carcinogens, while ochratoxin A and fumonisins are classified as Group 2B carcinogens, and deoxynivalenol and zearalenone as Group 3 (29). Thus, contamination with aflatoxin can be detrimental, and identifying and monitoring the aflatoxin-producing *A. flavus* in food systems is crucial.

In Nepal, the increasing incidence of liver cancer, particularly among rural populations such as farmers who rely heavily on maize as a dietary staple, raises serious concerns about chronic dietary exposure to aflatoxins, as well as highly toxic and carcinogenic metabolites produced by *Aspergillus flavus*. Although previous studies have reported aflatoxin contamination in maize and peanut samples from local markets, data on household-stored grains remain scarce. Moreover, most existing research has focused primarily on AFB1, with limited analysis on other types such as AFB2, AFG1, and AFG2. There is also a lack of detailed characterization of aflatoxin-producing *A. flavus* strains circulating in Nepal's diverse agroecological zones. In light of these gaps, this study aimed to identify and characterize aflatoxin-producing *A. flavus* in maize and peanut samples collected from various regions of Nepal, to provide knowledge for developing effective control and prevention strategies.

MATERIALS AND METHODS

Sample collection and storage. One hundred food samples (80 maize and 20 peanuts) were collected from various locations in Nepal, including Terai and Hilly regions (Kathmandu, Bhaktapur, Lalitpur, Hetauda, Kirtipur, Sankhu, Biratnagar, Chitwan, Surkhet, Dharan, Pokhara) from November 2022 to November 2024 adopting simple random method. The samples were collected in sterile ziploc bags, stored at 4°C, and transported to the Central Department of Microbiology, Tribhuvan University, for further laboratory analyses.

Estimation of moisture content. Two grams of each sample was placed in a petri dish and dried in a hot air oven at the temperature of 105°C for 4 hours, cooled in a desiccator, and weight loss was calculated as the percent of moisture content (33) as follows:

$$\% \text{ Moisture Content} = \frac{\text{Original weight - Final weight}}{\text{Original weight}} \times 100\%$$

Isolation and identification of fungi. For the isolation of fungi, the food sample was first surface sterilized with 1% sodium hypochlorite solution for 1 minute and inoculated in Potato Dextrose Agar (PDA) (Hi Media, India), then incubated at 28°C for 48 h. Fungi were purified either using the hyphal tip or single spore method in PDA. Further, pure colonies were identified based on morphology observed under microscope (26).

Phenotypic characteristics were confirmed based on the colony morphology in PDA and microscopic examination by Lactophenol Cotton Blue staining. Colonies exhibiting yellowish-green or dark green pigmentation with a white zone surrounding, which was later covered by conidia, were considered presumptive *A. flavus* isolates. The presumptive isolates were confirmed based on the characteristic features of septate branching hyphae, rough-walled conidiophores with a globose vesicle at the tip, phialides arranged in either uniseriate or biseriate patterns, and chains of rough-walled conidia originating from the phialides (26). The identified *A. flavus* isolates were preserved in PDA slants and stored at 4°C for further characterization.

Screening for aflatoxin-producing *A. flavus*. The suspected colonies of *A. flavus* were inoculated into the selective medium, *Aspergillus* Differential Medium (ADM) (Hi-Media, India). The isolates showing distinct yellow colonies were considered as aflatoxin producers (26).

Detection of aflatoxin production by *A. flavus*. Aflatoxin production was qualitatively analyzed by Thin Layer Chromatography (TLC) with silica gel coated in aluminum sheet (Merck, Germany) (27). Briefly, 6 log CFU/mL of *A. flavus* was inoculated into 100 g of maize and incubated at 28°C for 5 days. Aflatoxin was extracted from inoculated maize samples by the modified CB and Romer method (27, 28). To detect aflatoxin in TLC silica gel plate, plates were activated by heating at 80°C for one hour in a hot-air oven before use. About 5µL of both the standard AFB1 and the samples were then spotted onto the plates. The plates were placed into a tank containing a chloroform-acetone mixture (88:12 v/v) and left to develop for 30 minutes at room temperature. Then, the plates were examined under UV light (λ=260nm) to detect aflatoxins fluorescence. A blue fluorescence spots, corresponding to the standard AFB1, confirmed the presence of AFB1 in the sample (30).

Standard aflatoxin AFB1 and AFG1, AFB2 and AFG2 used in this study were purchased from Supelco (Bellefonte, PA, USA). The samples showing blue fluorescence spots under UV were further analyzed by HPLC equipped with a fluorescence detector (Shimadzu, Japan) to identify the types of aflatoxins produced. The column used for separation was a Shim-pack VP-ODS C18 column (250 × 4.6 mm) with temperature set at 50°C. Injection volume was 10 µL with the flow rate of 0.8 mL/min and the mobile phase used was water: acetonitrile: methanol (10:7:3, v/v/v). HPLC analysis was performed using a fluorescence detector at an excitation wavelength of 360 nm and an emission wavelength of 440 nm. Aflatoxin standards (AFB1-20 µg/mL, AFB2-3µg/mL, AFG1-3µg/mL, and AFG2-3 µg/mL) were used as references to compare the presence of aflatoxins in maize samples (29).

Statistical analysis. The statistical analyses were performed using IBM SPSS ver.27. The χ^2 and Fisher's tests were used to correlate the sample for fungal contamination.

RESULTS

Total moisture content in food samples. The moisture contents of different food samples obtained from the household and market were measured. Moisture contents of all the peanuts (market and household) and maize (market) samples were found within the recommended limit of the Codex Alimentarius Commission standard. However, the maize samples obtained from the household exceeded the recommended limit (Table 1).

Fungal contamination of food samples. Out of the 100 samples, 67% were contaminated with fungi. Fungal contamination was significantly higher in household-stored foods compared to market samples (p=0.00039). Peanut samples from households had the highest contamination rate (90.00%). However, the differences in contamination rates between maize and peanut samples were not significant (p=0.63, χ^2 =0.23) (Table 2).

Fungal diversity in food samples. Among the 67 fungal isolates, *A. flavus* was the most frequently isolated species in all the sample types, with the highest occurrence in household-stored maize (72.5%). The second most common were *Rhizopus* spp. and *Mucor* spp., with *Mucor* spp. being more common in household-stored peanuts (30.0%). Yeast contamination was minimal, detected only in maize samples (Table 3).

30% (15/50) of *A. flavus* isolates were preliminarily identified as aflatoxin producers. Notably, 40% (2/5) isolates from peanut samples were aflatoxin producers, whereas 28.90% (13/45) isolates from maize samples were aflatoxin producers. However, this difference was not significant (p=0.68; Table 4).

Screening of toxigenic *Aspergillus flavus*. Fifteen isolates produced yellow-orange pigment on ADM, indicating aflatoxin-producing *A. flavus* (Table 5).

Confirmation of Aflatoxin producing *A. flavus* and the type of aflatoxin. Out of 15 aflatoxin-producing *A. flavus* screened in ADM, 11 (73.33%) were confirmed by TLC. HPLC analysis revealed that Aflatoxin B1 was the most prevalent type, produced by *A. flavus* isolated from maize and peanuts samples from both market and household. Few of *A. flavus* isolates produced both aflatoxins B1 and B2, which were found in maize samples only. However, G1 and G2 were not detected in any of the samples (Table 6).

DISCUSSION

Our study indicates that fungal contamination in maize and peanut samples stored at the household continues to be a serious food safety issue in Nepal. The overall contamination rate of 67% reflects the ongoing problems linked to traditional storage practic-

Table 1. Total moisture content of samples

SN	Sample collection site	Sample type	No. of samples	Range of moisture content (%)	Mean ± SE (%)	Codex standard recommended limit (%)
1	Household	Maize	40	9.5 - 16.5	13.2 ± 1.1	< 15.5
2	Household	Peanut	10	4.5 - 9.5	7.0 ± 0.8	< 10
3	Market	Maize	40	9.5 - 14.5	12.1 ± 1.4	< 15.5
4	Market	Peanut	10	4.5 - 8.5	6.4 ± 0.9	< 10

Table 2. Fungal contamination rate in food samples collected from the market and households

S.N.	Sample collection site	Sample Type	Number of samples	Number of samples with fungal contamination (%)	p-value
1	Market	Maize	40	21 (52.5)	0.00039
2		Peanut	10	3 (30.0)	(χ^2 =18.25)
3	Household	Maize	40	34 (85.0)	
4		Peanut	10	9 (90.0)	

Table 3. Fungi isolated from different food samples

Types of fungi	Maize market (n=40)		Maize household (n=40)		Peanut market (n=10)		Peanut household (n=10)	
	No. of isolates	%	No. of isolates	%	No. of isolates	%	No. of isolates	%
Aspergillus flavus	16	40.00	29	72.50	1	10.00	4	40.00
Rhizopus spp.	2	5.00	3	7.50	2	20.00	2	20.00
Mucor spp.	2	5.00	1	2.50	0	0.00	3	30.00
Yeast	1	2.50	1	2.50	0	0.00	0	0.00
Total isolates	21	52.50	34	85.00	3	30.00	9	90.00

Table 4. Frequency of aflatoxin-producing *A. flavus* in different samples

Type of sample	Isolation Source	*A. flavus* isolates		Aflatoxin-producing *A. flavus* isolates		p-value calculated using Fisher's exact test
		Number	Percentage (%)	Number	Percentage (%)	
Maize	Market	16	32.00	5	31.25	
	Household	29	58.00	8	27.59	0.68
Peanut	Market	1	2.00	1	100.00	
	Household	4	8.00	1	25.00	
	Total	50	100.00	15	30.00	

es, a result that is consistent with earlier reports from Nepal and neighboring South Asian countries. The greater contamination observed in household-stored samples likely results from longer storage durations, poor aeration, and fluctuations in humidity and temperature, after harvest. Joshi et al. (3) also reported extensive mold contamination in maize samples collected from different places of Nepal and linked it to inadequate post-harvest handling and storage conditions. Similar studies from India have reported

higher fungal loads in grains stored at homes than market samples, underscoring how the storage environment and post-harvest management practices affect contamination (30). In Iran, Rahmani et al. (14) found that 21.5% of rice samples were contaminated with *A. flavus*-derived AFB_1, with total aflatoxin levels ranging from 0.1 to 5.8 ng/g in rice samples with some exceeding the EU standard of 2 ppb. Although both maize and peanuts were susceptible to fungal invasion, household-stored peanuts showed notably higher contamination, suggesting greater vulnerability under certain storage situations. In Nepal, many farmers keep maize and peanuts in jute sacks or bamboo bins, often in damp places. Such settings raise humidity and favor insect and pest invasion. In contrast, market sold products are generally handled more carefully and kept for shorter durations, which minimizes the risk of fungal contamination as well as pest and insect infestations. This observation aligns with findings from Bangladesh, where Rahman et al. (31) noted that peanuts exhibited greater fungal diversity and aflatoxin risk compared to other staple foods (31). Amiri et al. (15) also confirmed that cereal-based food such as bread flour was contaminated with AFB_1 before heat treatment, indicating the invasion of aflatoxin-producing *A. flavus* in staple grains and a high risk of AFB1 contamination. However, in our study the overall difference in contamination rates between the two commodities did not indicate statistical significance, suggesting that other factors such as the duration of storage, moisture content, and handling practices might modulate the contamination risk in a complex manner.

Table 5. Toxigenic *Aspergillus flavus* on ADM

Organism code	Production of yellow-orange pigment on ADM	Intensity of yellow-orange pigment on ADM
AS-1	+	+ +
AS-2	+	+
AS-3	+	+
M-1	+	+
M-2	+	+
M-3	+	+
M-4	+	+
M-5	+	+
M-6	+	+
M-7	+	+
M-8	+	+
M-9	+	+
AS-4	+	+
AS-11	+	++
AS-5	+	+

The predominance of *A. flavus* among the fungal isolates is of particular concern given its well-documented capacity for aflatoxin production. According to our findings, *A. flavus* was the most frequently isolated fungus, especially in household-stored maize, which is in agreement with previous studies in South Asia, where this specie has consistently been identified as the major aflatoxin producer (32, 33). In addition to *A. flavus*, other genera such as *Rhizopus* spp. and *Mucor* spp. were also detected but at lower frequencies. This diverse fungal profile in household-stored samples suggests that the storage environment may foster a complex mycobiota, thereby potentially exacerbating the risk of multi-mycotoxin contamination.

A noteworthy aspect of our study is the identification of aflatoxin-producing *A. flavus* isolates. Approximately 30% of the *A. flavus* isolates were identified as potential aflatoxin producers using a selective medium, and a substantial proportion of these were subsequently confirmed by chromatographic techniques. The predominance of AFB1, along with the occasional detection of AFB2 in maize samples,

Table 6. Detection of Aflatoxin producing *A. flavus* and identification of aflatoxin type

Source of sample	Number of Aflatoxin producing *A. flavus* detected by		Type of aflatoxin identified by HLPC (Number of *A. flavus* isolate)
	Aspergillus Differential Medium	Thin Layer Chromatography	
Maize market	5	4	B1(3), Both B1 and B2(1)
Maize household	8	5	B1 (4), Both B1 and B2 (1)
Peanut market	1	1	B1 (1)
Peanut household	1	1	B1 (1)
Total number	15	11	11

aligns with findings from other regional studies, which consistently report AFB1 as the most common and toxic aflatoxin in contaminated grains (3). These results emphasize that the risk associated with aflatoxin exposure in Nepal is not only due to the presence of fungal contaminants but also due to the specific toxigenic profiles of the isolates.

The differences in aflatoxin profiles between maize and peanut samples observed in our study may reflect crop-specific interactions with fungal communities. While both commodities are susceptible to aflatoxin contamination, environmental factors, crop physiology, and storage practices could influence the biosynthesis of specific aflatoxins. This observation is supported by studies from India, where similar variations in aflatoxin types were noted between different agricultural products (30, 34). While fungal pathogens in clinical settings in Nepal are well characterized, food borne fungi are less emphasized (34).

Overall, findings of the current study underscore the urgent need for improved storage practices and regular monitoring of fungal contamination and mycotoxin levels in staple foods. Public awareness campaigns, adoption of better post-harvest handling strategies, and stricter regulatory frameworks are essential to mitigate the public health risks posed by aflatoxins to ensure food safety. In addition, low-cost, eco-friendly biological control strategies, such as the use of probiotic bacteria and yeasts represent promising method for reducing mycotoxin contamination in food grains and minimizing public health concerns (15, 16). Future research should focus on elucidating the influence of seasonal variations and climate conditions on fungal proliferation and mycotoxin production, which would aid in developing targeted intervention strategies for South Asia.

CONCLUSION

Our findings indicate that maize and peanut are prone to contamination with aflatoxin producing *A. flavus*, which is predominant in household-stored samples, raising the concern of food safety in Nepal. To address this concern, well-managed environmental storage conditions, followed to strict regulatory standards like those from the FDA and Codex, can help minimize contamination with aflatoxin-producing fungi. It is also equally important to enforce strict regulatory mechanisms and implement regular monitoring programs to mitigate aflatoxin contamination in cereal-based products, raise public awareness on safe storage conditions and health risks, and promote eco-friendly interventions such as the use of probiotic strains to control the growth of aflatoxin-producing molds.

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