

Determination of the incidence of *Aspergillus* species isolated from maize kernels and maize based products in Jammu and Kashmir, India.

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Abstract: Fungal contamination and Aflatoxin (AF) poisoning among maize and maize based products continues to exacerbate the food security crisis in India including temperate Jammu and Kashmir. This study determined the distribution and contamination levels of *Aspergillus* spp. in maize and maize-based products. Maize grain samples (n= 455), semi-processed grain (n= 115), flour (n= 36), were collected during the 2013 and 2014 growing seasons. *Aspergillus* spp. were isolated by growing on Potato Dextrose Agar (PDA) and Malt Extract Agar (MEA). Fungal species commonly isolated from the collected samples included *Aspergillus parasiticus*, *A.niger*, *A. flavus*, *A. nomius*, *A. tamari* and *A. caelatus* with *A. flavus*, *A. parasiticus* and *A. niger* being most frequent in selected samples. The frequency of *A. niger* was higher in semi-processed grain than in whole grain and packed flour samples. The high temperature and periodic drought prevalent in the semi-arid regions could explain the higher levels of *A. flavus*. In addition, unfavourable drying and storage practices may aggravate the problem. Therefore, it is recommended that the careful monitoring of AF be continued.

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1. Introduction

Food microbiology is an untouched field in Kashmir valley where majority of population derive their livelihood from agriculture and other related practices. In food microbiology, the utmost importance is the study of microorganisms involved in food spoilage and can risk human health (Fratamico PM and Bayles DO, 2005). The pathogens deteriorating the food items are the leading causes of illness and death in undeveloped countries, accounting approximately 1.8 million deaths annually (Faruque, SM, 2012). The natural fungal flora associated with foods is dominated by three genera of fungi; *Aspergillus*, *Fusarium*, and *Penicillium*, which except for the *Fusarium* plant pathogens, may include commensals as well as pathogens with majority of *Aspergillus* species being pathogenic. *Aspergillus flavus*, *A. parasiticus* and *Aspergillus niger* are the most important aflatoxigenic/pathogenic species naturally occurring in agricultural commodities (Pitt and Hocking, 1997). Distributed worldwide in soil and air, these species can primarily colonize plants in the field and secondarily transfect harvested or stored plant products. Maize and walnuts are the common agricultural products commonly consumed in Kashmir. However, maize, maize based products and walnuts are vulnerable to degradation by mycotoxigenic fungi belonging to *Aspergillus*,

Fusarium and *Penicillium* genera. Contamination of maize by these fungi renders the grain unfit for human consumption due to discolouration, reduction in nutritional value and most importantly the production of Mycotoxins. The potential for spoilage and mycotoxin production depends upon the types of fungi present, the composition of the food and the conditions of handling and storage. For example, dried foods are susceptible to spoilage and toxin production if storage temperature is suitable for fungal growth (Misra, 1981).

The present study was carried out to examine the *Aspergillus* population in food samples (maize and maize based products) from different regions in Kashmir and to find various species belonging to genus *Aspergillus*.

2. Material and Methods

Sample collection

Sampling was carried out in three different regions of temperate Kashmir valley namely, north, south and central districts of Kashmir during the 2013 and 2014 cropping seasons (Table 1). Two kilograms of each of the following were collected; whole maize grain (455 samples), semi-processed grain (115 samples) and maize flour (36 samples). The samples were collected from farmers and traders (shops and markets) in each of the three regions. Packed maize flour samples were collected from shops, markets and

farmers in the same regions. The samples were packed in sterilized polythene bags and stored at 4°C until analysis. Fungal isolation and culturing was done at Microbiology and Pathology laboratory, Centre of Research for Development, University of Kashmir, Srinagar, India.

Isolation and identification of fungi

Isolation of fungi by direct plating method

The method used for isolation of fungi was previously described by Abdullah *et al.* (2002). Twenty grams of each sample were surface sterilized by 9% sodium hypochlorite solution (NaOCl) in a sterile conical flask for 2-3 min, and then washed by distilled water 3 times for 1-2 min to removing the toxic activity of the chemical agent on the samples. The disinfected samples transferred with sterile forceps into Petri dish contain sterilized Potato Dextrose Agar (PDA), at the rate of (4-6) pieces per plate, depending on the size of the particles, larger samples cut into small pieces. PDA supplemented with 0.5 mg Streptomycin/ml to restrict bacterial growth. Three replicates were made and the plates were incubated at 25°C for 5-7 days. Fungal colonies were identified according to morphological and microscopic characteristics.

Isolation of sample surface mycoflora

The collected samples were directly cultured without surface sterilization at the rate of 6-8 samples/Petri plate into Petri dish containing sterilized malt extract agar (MEA) and Potato Dextrose Agar (PDA). Three replicates were made and the plates were incubated at 25°C for 5-7 days. Fungi colonies were identified according to morphological and microscopic characteristics (Pitt *et al.*, 1992).

Isolation by Standard dilution method

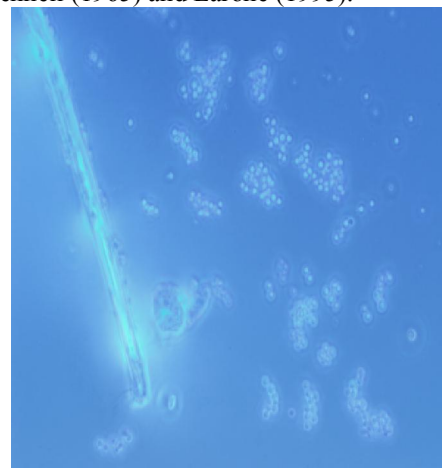
Standard dilution method was used to determine total fungal count in maize flour. Twenty grams of each composite sample (fine powder) were transferred into 250 ml conical flask containing 100 mL of sterile distilled water and were mechanically homogenized at constant speed for 15 min using (model). The sample-water suspension was allowed to stand for 10 min with intermittent shaking before being plated. Appropriate tenfold serial dilutions (1:10) were prepared and 1 ml portions of suitable dilutions of the resulting samples suspension were used to inoculate Petri dishes each containing 20 ml Potato Dextrose Agar (PDA) and Malt extract Agar (MEA). Plates were then incubated for 7 days at 28°C. Three replicates plates per medium were used for each sample and the developing fungi were counted and the number per mg dry sample was determined and identified according to several key processes. Data expressed are average of all these media. After incubation, the results were expressed in Colony-Forming Units (CFU) /g of samples; all plates were

examined visually, directly and with a microscope (Anonymous, 1996).

Fungal Species Identification and Counting of Colonies

Pure colonies on PDA and MEA agar medium were sub-cultured onto the Czapek yeast extract agar (CYA; 1 g K₂HPO₄, 10 ml Czapek concentrate, 5 g powdered yeast extract, 30 g sucrose, 15 g agar), whose pH was adjusted to 7.2 and the plates incubated at 30°C for 7 days. Species of *Aspergillus* were identified based on cultural and morphological characteristics including colony colour, size of sclerotia, texture and conidial morphology characteristics (M. A. Klich, 2002), and by comparison with reference strains Sheri Kashmir University of Agricultural Science (SKUAST) Kashmir. The reference cultures were subcultured at the same time of plating the maize samples. Colonies of other isolated fungal pathogens were identified to genus level. The colony forming units (CFU) of each fungal species were counted using Remis colony Counter. Equation (a) was used to determine the population.

Flour samples were serially diluted in sterile distilled water and 1 ml of the 10⁻³ and 10⁻⁴ dilutions was applied to plated Czapek Dox agar. The isolation plates were incubated at 25 °C for 14 days. The number of kernels showing growth of *Aspergillus* species and the number of *Aspergillus* colonies per dilution plate were determined. The number of colony forming units per gram (CFU/g) of soil and flour samples was calculated by multiplying the number of colonies by the respective dilution factor. Isolates of *Aspergillus* spp. were sub-cultured on Czapek Dox agar, incubated for 7 to 14 days at 25 °C and identified to species level according to Gao et al (2007), Raper and Fennell (1965) and Larone (1995).

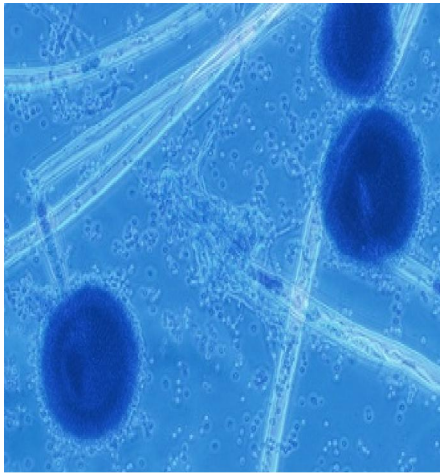


a. Dispersed conidia

(CFU/g peanuts) of the fungal species.

$$CFU/g = \text{Colony Counts/volume plated} \times \text{dilution factor}$$

The volume plated was 10 ml while the dilution factors were 0.25 for the first dilution (10^{-1}) and 0.025 for the second dilution (10^{-2}).



c. Conidiopores

Fig. 1 Microscopic features of fungal spores.

The The fungal strains isolated from 445 maize samples (whole grain, semi-processed and maize flour shelled) collected from farmers and markets from three regions of Kashmir are shown in Table 1. The specific *Aspergillus* spp. isolated from whole and unprocessed maize grain were *A. flavus*, *A. niger*, *A. fumigatus*, *A. versicolor*, *A. terreus*, *A. clavatus* and *A. ochraceus*. The most frequently isolated were *A.*

flavus and *A. niger*, while *A. tamari* was the least frequently isolated *Aspergillus* species and was mainly isolated in samples from the central Kashmir (Tables 2). Higher *Aspergillus* spp isolation frequencies were recorded in grain samples from south and central Kashmir region than those from north Kashmir probably due to low temperature in this region. Grain samples collected from farmers in the central and north region had higher incidences of *A. flavus*, of up to 19% compared to grain from traders.

3. Results

The semi-processed grain and flour samples contained the highest (25%) incidence of *A. flavus* (Figures1; Table 2). Other *Aspergillus* spp. isolated from processed grain and flour samples were *A. fumigatus*, *A. niger*, *A. terreus* and *A. versicolor*. Maize flour collected directly from farmers contained higher levels of *A. flavus* with up to 6.9×10^3 CFU/g, than packed flour bought from shops and markets (Figure 3).

The mean counts of the fungal colonies ranged from 4×10^2 to 16×10^3 cfu/g. Whole maize grains and semiprocessed maize from farmers were highly contaminated by total fungi. The commonly isolated fungi were species of *Aspergillus*, *Penicillium* and *Mucor*. The most represented genus in contaminated samples was *Aspergillus*, which was isolated in all analyzed samples with the mean percentage of 64.0%.

Table 1. Type of samples collected from farmers and traders (shops and markets) in Kashmir

Sample Type	South Kashmir		Central Kashmir		North Kashmir	
	From Farmers	From Traders	From Farmers	From Traders	From Farmers	From Traders
Whole grain	50	60	40	30	55	35
Grain From stores	60	40	30	25	20	40
Semi Processed grain	40	30	-	20	25	-
Flour in packed bags	-	10	-	5	6	8
Flour in open bags	-	5	-	2	-	-

Table 2. Percent incidence of different Aspergillus species isolated from infected maize grain, semi-processed maize grain and processed maize grain from south, central and north Kashmir

Sample	Type	Source	Region	<i>A. parasiticus</i>	<i>A. niger</i>	<i>A. flavus</i>	<i>A. fumigatus</i>	<i>A. ochraceous</i>	<i>A. vesicolor</i>	<i>A. tamari</i>
Grain	Traders	Central		7	12	8	4	0.5	0.2	0.1
Grain	Traders	South		8	9	4	2	0.8	0.6	0.0
Grain	Farmers	Central		5	11	6	0.9	0.3	0.4	0.2
Grain	Farmers	North		9	5	2	-	0.9	0.2	0.0
Grain sp	Traders	Central		2	5	-	0.6	-	-	-
Grain sp	Farmers	South		14	9	2	-	0.1	-	0.0
Grain p	Traders	North		3	2	1	-	-	-	-

SP= semi processed, P= processed, South= south Kashmir, North= north Kashmir, Central= central Kashmir

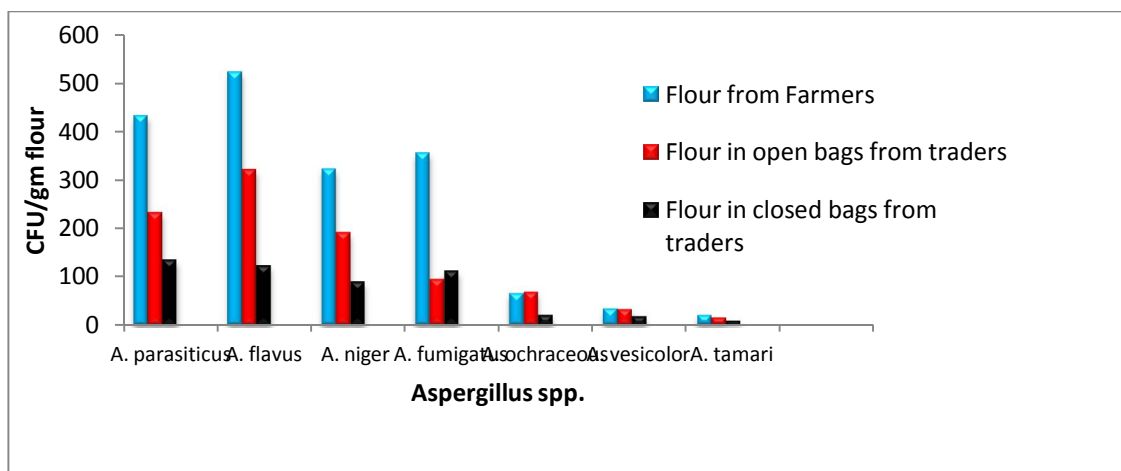


Figure 1. Mean number of colony forming units (CFU/g) of *Aspergillus* species isolated from maize flour collected from farmers and traders in Kashmir

4. Discussions

This study provides the comprehensive documentation of the distribution of *Aspergillus* species maize and maize based products in Kashmir valley. The commonly isolated fungi were species of *Aspergillus*, *Penicillium* and *Mucor*. The most represented genus in contaminated samples was *Aspergillus*, which was isolated in all analyzed samples with the mean percentage of 64.0%. The semi-processed grain and flour samples contained the highest (25%) incidence of *A. flavus*. Other *Aspergillus* spp. isolated from processed grain and flour samples were *A. fumigatus*, *A. niger*, *A. terreus* and *A. versicolor*. Higher *Aspergillus* spp isolation frequencies were recorded in grain samples from south and central Kashmir region than those from north Kashmir probably due to low temperature in this region. Grain samples collected from farmers in the central and north region had higher incidences of *A. flavus*, of up to 19% compared to grain from traders.

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