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INCIDENCE AND MOLECULAR DETECTION OF OCHRATOXIGENIC FUNGI FROM INDIAN CEREAL GRAINS

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ABSTRACT

A total of 150 cereal samples from three regions of Southern India i.e. Karnataka, Andhra Pradesh and Tamilnadu were collected during winter 2010 – 2012 to determine the ochratoxigenic fungal incidences. A total of 150 fungal isolates comprising, *Aspergillus ochraceus, A. niger* and *Penicillium verrucosum* were recovered and tested for ochratoxin A (OTA) production. Chemotype determination of fungal isolates was carried out by molecular and chemical analysis through PCR and HPLC methods. Out of 3 study sites the incidence of ochratoxigenic *Aspergillus* and *Penicillium* species were recorded as 30%, 33% and 22 % in Karnataka, Andhra Pradesh and Tamilnadu regions respectively. Based on PCR results *A. ochraceus* (36), *A. niger* (29) and *P. verrucosum* (38) were recorded as OTA chemotypes. The mean concentrations of OTA were determined as 12.2 µg/kg, 15.6 µg/kg and 8.3 µg/kg for wheat, maize and rice, samples respectively. The results of the present study suggest that the PCR developed can be used to determine ochratoxigenic fungal contamination in agricultural commodities such as cereals and other food grains. Moreover, the high levels of OTA contamination recorded in present study warrants that there is a need to undertake mycotoxin awareness creation programme among the different cereal growing regions of India to reduce risk of its harmful effects to human and other farm animals.

KEYWORDS: **Fungi; Ochratoxin A; cereals; PCR; HPLC.**

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INTRODUCTION

Ochratoxins are a group of toxic secondary metabolites produced by certain fungi in the genera *Aspergillus* and *Penicillium*[1] of which ochratoxinA (OTA) is the most toxic. [2,3] The health effects of OTA include nephrotoxic, hepatotoxic, teratogenic, mutagenic and immunosuppressive disorders, and also OTA was classified as a group 2B human carcinogen by the IARC.[4,5] Though OTA is considered to be the major causative factor in mycotoxic porcine nephropathy (MPN) in many european countries[6] the role of OTA in a similar human disease (BEN, balkan endemic nephropathy) and in human renal urothelial tumors is still not clear.[7] Creppy *et al.* [8] reported the presence of OTA in foods of plant origin, animal tissues, milk, blood and other organs. Extensive surveys conducted in different countries further revealed the presence of OTA in food stuffs such as cereals, coffee beans, pulses, spices, meat and cheese products.[9] Given its existence in several consumer products, consumer exposure to OTA is increasing. But in India there is no such standards was implemented for OTA level in foods. Given its existence in several consumer products, consumer exposure to OTA is increasing. In order to protect consumers, The European union (EU) has defined regulatory limits of OTA for the food products intended to human consumption, i.e., 10 ppb in dried vine fruits and instant coffee, 5 ppb in cereals and roasted coffee and 2 ppb in wine (EC 2002) [10]. But in India there is no such standards was implemented for OTA level in foods.Over the past two decades, mycotoxicosis has been observed both in human and animals. Mycotoxicosis causes acute liver damage, liver cirrhosis, induction of tumors, attack on central nervous system, skin disorders and hormonal defects.[1] Unfortunately, no organized efforts in past has been made to address this issue in India. In India scanty reports have been recorded on ochtaoxin contamination in coffee and other species.[11] To best of our knowledge this is the first report of OTA contamination in Southern Indian cereal grains.In the present study PCR assays were developed and used to monitor the ochratoxin producing fungi incidence in Southern Indian cereal grains. The levels of OTA contamination was quantified by HPLC analysis.

MATERIALS AND METHODS

(i) Sample collection

Infested cereal samples comprising maize, wheat and paddy were cllected from high rain fall areas in Southern India such as, Karnataka, Andhra Pradesh and Tamilnadu. A total of 150 samples (50 samples from each region) were collected during winter 2010-2012. All the samples were in uniform size of 250 grams of each was collected and stored at 4 ^{o}C for further analysis of ochratoxigenic mycoflora and toxin detection.

(ii) Isolation and identification of moulds

The counts of fungal populations and dominant genera were isolated from the cereal samples. Grains were surface sterilized and placed onto PDA and the pure cultures were maintained on Czapeck Dox agar medium. To identify the fungi at the genus level, the Manual of Barnett and Hunter[12] was used. The reference strains (Table-1) were obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India was used as a standard cultures. Our morphological findings were further confirmed by species specific PCR assays.

Table 1
Standard strains used in this study and their toxin chemotypes.

S.No	STRAIN NAME	SOURCE	PCR	HPLC
1	*Aspergillus niger 236*	ITCC	+ve	+ve
2	*Aspergillus niger* 1436	ITCC	+ve	+ve
3	*Aspergillus ochracious* 3167	ITCC	+ve	+ve
4	*Aspergillus ochracious* 2454	ITCC	+ve	+ve
5	*Aspergillus ochracious* 1810	MTCC	+ve	+ve
6	*Penicillium verrucosum* 2156	ITCC	+ve	+ve
7	*Penicillium verrucosum* 2986	ITCC	+ve	+ve
8	*Penicillium verrucosum* 1758	MTCC	+ve	+ve
9	*Penicillium veridicatum* 2007	MTCC	+ve	+ve
10	*Aspergillus flavus 46283*	*ATCC*	-Ve	-Ve
11	*Aspergillus flavus 152*	*NCIM*	-Ve	-Ve
12	*Aspergillus flavus 645*	*NCIM*	-Ve	-Ve
13	*Aspergillus flavus 650*	*NCIM*	-Ve	-Ve
14	*Aspergillus flavus 2798*	*MTCC*	-Ve	-Ve
15	*Aspergillus parasiticus 2797*	*MTCC*	-Ve	-Ve
16	*Fusarium graminearum 2089*	*MTCC*	-Ve	-Ve
17	*Fusarium verticelloides 3693*	*MTCC*	-Ve	-Ve
18	*Fusarium sporotrichoides 2081*	*MTCC*	-Ve	-Ve
19	*Penicillium chrysogenum 6479*	*MTCC*	-ve	-ve
20	*Fusarium moniliforme 156*	*MTCC*	-ve	-ve

(iii) Molecular detection of toxigenic fungi

Extraction of DNA

DNA was extracted from pure cultures of fungi using DNeasy plant Minikit (Qiagen, Gambh, Germany, Cat-69106). A 10 ml of sterile potato dextrose broth (PDB) was inoculated with 500 µl of the single-spore culture suspensions and flasks were incubated under agitation (100 rpm) at room temperature for 3 days. The fungal cultures were then filtrated in a polyethylene membrane and washed twice with sterile distilled water. The membrane with the mycelium was folded and quickly dried with a tissue paper. The near dried fungal mycelium was transferred to an Eppendorf tube, frozen for 2 h at -20°C and lyophilized over night. Mycelial mat (100 mg) was ground in liquid nitrogen and total genomic DNA was extracted as per manufacture's instructions. Nuclease free water was used to dilute the DNA stock solution and the concentration was estimated by using Nanodrop (Thermo Scientific nanodrop 2000c) and DNA was stored at -80 ^{o}C until use.

Species specific PCR assays

Species specific PCR assays were carried out for all the isolates using reported primers specific to major ochratoxigenic fungal species such as *A. niger, A. ochraceus* and *P. verrucosum.* Primer sequences and references are given in Table-2.

Table 2
Primers used in present study.

Primer	Sequence (5'-3')	Amplicon size	Reference
OCRA1	CTTCCTTAGGGGTGGCACAGC	400 bp	Patino et al. [13]
OCRA2	GTTGCTTTTCAGCGTCGGCC		
ITS1	TCCGTAGGTGAACCTGCGG	420 bp	Gonzalez-Salgado et al.[14]
NIG2	AAAGTCAATCACAATCCAGCCC		
Otpkspv for	CCGCAGGAAGGATCACGAAGAA	415 bp	Schmidt-Heydt et al. [15]
Otpkspv rev	TCCTACTTCCCCTGCTGCAAATCA		
AO pks1	C AGA CCA TCG ACA CTG CAT GC	549 bp	This work
Aopks2	CTG GCG TTC CAG TAC CAT GAG		
Penpks1	GT CTT CGC TGG GTG CTT CC	397 bp	This work
Penpks2	AG CAC TTT TCC CTC CAT CTA		

(iv) Detection of ochratoxigenic fungal species

Primers and PCR conditions:

Two pairs of primers were designed and used for specific detection of ochratoxigenic fungi by targeting the metabolic pathway genes (*pks*)

specific to toxin chemotype. The homology searches were done using the BLAST programme and highly specific regions were considered for primer design. Primer sequences and targeted amplicon sizes were listed in Table-2.

PCR assay for detection of mycotoxigenic molds

PCR was carried out in an Eppendorf master cycler gradient (Hamburg, Germany, Mastercylcer^R pro 384) with a reaction volume of 30 µl. The amplification mixture consisted of template DNA (1.0 µl), MgCl2 (2.0 mM; Sigma, Cat-M8787), 1X PCR buffer (Sigma, Cat-P2317), dNTP mix (200 µM; Sigma, Cat-D7295), *Taq* polymerase (1.0 unit; Sigma, Cat-D40724) and primer pairs specific to the targeted genes (species specific PCr assays and toxin chemotypes) were added at a concentration of 100 nM to the each individual reaction. The PCR cycling conditions were carried out with an initial denaturation at 94 ^oC for 4 min, followed by 30 cycles of 94 ^oC for 1 min, 58 ^oC for 1 min and 72 ^oC for 1.5 min with a final extension of 72 ^oC for 8 min. After successful amplification, PCR products were loaded onto 1% agarose gel containing ethydium bromide and visualized under UV.

(v) Chemical analysis of OTA by HPLC

Toxin Extraction and Cleanup from isolated fungi:

To assess the degree of contamination of the cereal grains, agar plating was carried out. Twenty-five grains of cereal sample were disinfected with sodium hypochlorite solution (0.4% w/ v) and grains were placed (10-15 grains in every plate) directly on the surface of Yeast Extract Agar (YEA). Plates were incubated at 28 ^oC for 7 days and the growth of the cultures was monitored visually. Presumptive colonies of *Aspergillus* and *Penicillium*, according to their coloration and form, were isolated for tests of OTA production in culture media. Five microliters of the suspension (3 x10^6 of conidia ml^{-1}) for each strain isolated were placed in the centre of a dish of YEA. After seven days of incubation at 28 ^0C, direct extraction was carried out from three agar discs taken from the centre of the colony. Extraction was carried out in 2.5 ml of methanol/formic acid (25:1, v/v) for 15 min in an ultrasound bath. A strain of *A. ochracious* OTA producer in MEA, belonging to MTCC culture collection, was used as positive control for OTA production.

From contaminated cereal grains

OTA was extracted by using an adaptation of the extraction procedure of the Yu *et al.*[16] Powdered cereal samples (50 g) were transferred into a 500 ml PTFE (polytetrafluoro ethylene) container mixed with 200 ml chloroform and 20 ml 0.1 M orthophosphoric acid. After centrifugation for 10 min under 3000 rpm at 5-10 ^0C, the chloroform phase was transferred to a 500 ml beaker. The remaining part was extracted again with 200 ml chloroform and 20 ml of 0.1 M orthophosphoric acid. The combined chloroform phases (ca 350 ml) were evaporated to dryness by rotary evaporation at 30-40 ^0C. The residue was dissolved in 100 ml of 0.5 M NaHCO3 and transferred to a 120 ml PTFE container. After 10 min centrifugation at 3000 rpm at 5-10 ^0C, 20 ml of sample was passed through an Ochratest^{TM} immunoaffinity column (Vicam, Watertown, MA, USA). Before loading the extract, the Ochratest^{TM} column was conditioned with PBS, pH 7.4 (20 ml). Twenty milliliters of de-ionized water was used to wash the loaded immunoaffinity column and OTA was then eluted with 2 ml methanol and 2 ml de-ionized water. One millilitre sample of elute was then filtered through a 0.45 µm microfilter (Millex1-HV) for HPLC analysis.

HPLC analysis

HPLC was carried out as per Nesheim *et al.*[17] Briefly, 50 ml samples were injected by full loop injection. The chromatographic system consisted of a Perkin-Elmer LC049 isocratic pump (Norwalk, CT, USA) equipped with a Rheodyne model 7125 NS injection valve (50 ml) (Rheodyne, Cotati, CA, USA), an RF-551 fluorescence spectrophotometer detector (Shimadzu, Kyoto, Japan) equipped with a 150W xenon lamp. The mobile phase was acetonitrile: water: acetic acid (45:54:1 v/v/v) eluted at a flow rate of 1.0 ml/min. OTA was assayed by measuring peak height at the OTA retention time and comparing it with the relevant calibration curve (five points, in the

range 1-5 ng of ochratoxin A/ml, r^2 0:9992). Standard solutions for the calibration curve were prepared in the mobile phase from a stock solution containing 10 µg/ml of OTA in toluene: acetic acid (99:1).

(Vii) Statistical analysis

Incidences of fungal diversity was quantitatively analyzed for density, frequency, and abundance.[18] The species richness, diversity index (H) and concentration of dominance (Cd) of the species in the sampling area were also analysed. The diversity index was calculated following Shannon & Wiener[19] method as: $H = -\Sigma(Ni / N) \log_2 (N i /N)$.

The concentration of dominance was determined using the Simpson's index[20] as:

$Cd = \Sigma (Ni /N)^2$

Where, N_i is number of individual species and N is total number of all species present in the studied area.

RESULTS

1. Mould incidence and diversity in samples

A total number of 120 cereal samples were collected randomly from different fields of Southern India, during winter 2010-20102. Table-3 showed diversity of fungal species including frequency, density and the diversity indices like important value index (IVI), Shannon index (species richness) and Simpson index (Diversity of the species). Across the study area, *Fusarium, Aspergillus* and *Penicillium* were identified as most predominant genera with several species. It was also observed that, out of 3 study sites the incidence of ochratoxigenic *Aspergillus* species was recorded as 32%, 33% and 12 % in Karnataka, Andhra Pradesh and Tamilnadu respectively. *A. ochraceus* and *A. niger* were observed with 100% frequency in Karnataka; whereas in Andhra Pradesh, *P. verrucosum* and *A. niger* showed 100% frequency (Table-3). *A. ochraceus* and *P. verrucosum* were less frequent and highly densed in Tamilnadu with IVI of 52.6 and 59.7, respectively. The frequency and diversity of identified fungal species in Karnataka and Andhra Pradesh were significantly correlated ($p< 0.05$), however the fungal isolates of Tamilnadu were non-significantly correlated with other two regions ($p> 0.05$). The concentration of dominance (Simpson index) was higher in Tamilnadu when compared with other regions of the study (Table-3).

Table 3
Incidence of ochratoxigenic Fungal species.

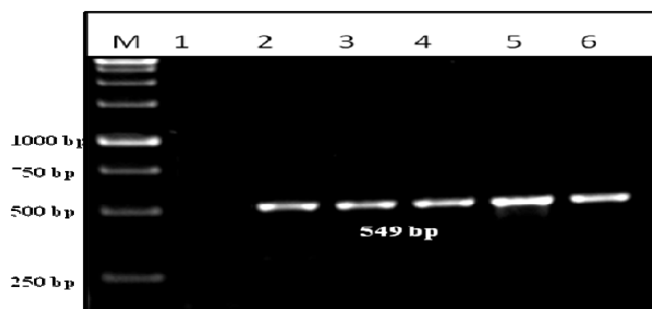
Name of the organism isolated	Karnataka			Andhra Pradesh			Tamilnadu		
	Fr	Den	IVI	Fr	Den	IVI	Fr	Den	IVI
A.niger	100.00	1.40	22.2947	100.00	1.00	17.0804	40.00	0.40	12.4275
A.ochraceus	100.00	0.40	6.16667	40.00	1.00	16.4671	0.00	0.00	0.0
OtherAspergillus species	20.00	0.20	6.56765	40.00	0.80	13.901	40.00	0.40	12.4275
P. verrucosum	80.00	1.60	23.302	100.00	0.80	13.5675	20.00	0.20	7.3766
Other Penicillium species	60.00	0.60	12.7343	40.00	0.60	11.3348	40.00	1.00	19.5304
Shannon index	**2.53047**			**2.6795**			**2.218**		
Simpson index	**0.0886**			**0.0746**			**0.150**		

2. Mycotoxigenic potential of fungal species collected from cereal samples

DNA was isolated from pure cultures of fungi isolated from cereal samples and subjected to PCR using group specific primers and further confirmed with toxin analysis by HPLC. The PCR and HPLC analyses of standard strains showed similar results for toxin chemotypes (Table-1). Target gene amplifications were represented in Fig-1A and 1B for ochratoxigenic *Aspergillus* and *Penicillium* respectively. A total of 150 fungal isolates, comprising *A. ochraceus* (45), A. niger (56) and *P. verrucosum* (49), were tested for ochratoxinA by PCR and HPLC methods. Based on PCR results *A. ochraceus* (36), *A.*

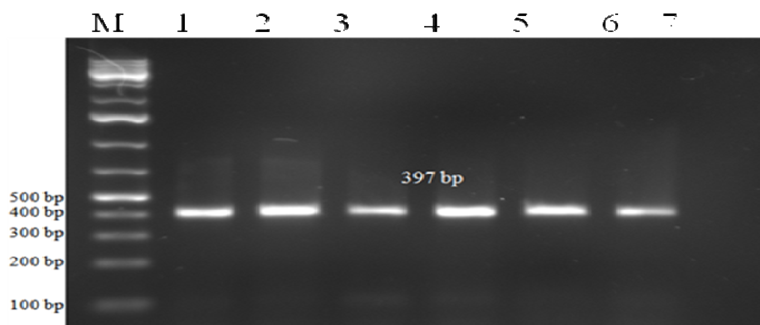
niger (29) and *P. verrucosum* (38) were recorded as OTA chemotypes.The quantitative analysis of OTA was carried out by HPLC. All the isolates of *A. ochraceus* and *P. verrucosum* were stayed as positive for production OTA, whereas, out of 29 PCR positives isolates *A. niger,*19 only showed positive signal for OTA production *in vitro*.

Figure 1A
Detection of ochratoxigenic Aspergillus species.



LaneM- 1 kb DNA ladder, 1- negative control, lane 2- OTA producing Aspergillus standard strain, Lane 3-6 OTA positive strains of Aspergillus spp.

Figure 1B
Ochratoxigenic Penicillium species.



LaneM- 100 bp DNA ladder, lane 1- OTA producing Penicillium verrucosum standard strain, Lane 2-6- OTA positive strains of Penicillium spp., Lane-7 -negative control.

3. Mycotoxin quantification

Thirty out of 50 wheat samples contained OTA in the range of 4.604 - 12.101 µg/Kg, with a mean value of 8.2 µg/Kg. Thirty seven out of 50 maize samples were found to be OTA positive, ranging from 3.3 to 27F.021 µg/Kg. Maize samples had the highest mean level of 15.6 µg/Kg and the highest concentration was observed as 42.00 µg/Kg in the present study. Twenty out of 50 rice samples were positive for OTA, ranging from 4.9 to 12.67 µg/Kg, with a mean level of 8.3 µg/Kg (Tabl-4). Out of studied cereal varieties rice samples were moderately contaminated with OTA when compared with wheat and maize. All these HPLC chromatograms were compared with OTA standard (Fig-2A), chromatographic representation of cereal samples was showed in Fig -2B.

Figure 2A
HPLC chromatogram Ochratoxin A standard (Sigma, India).

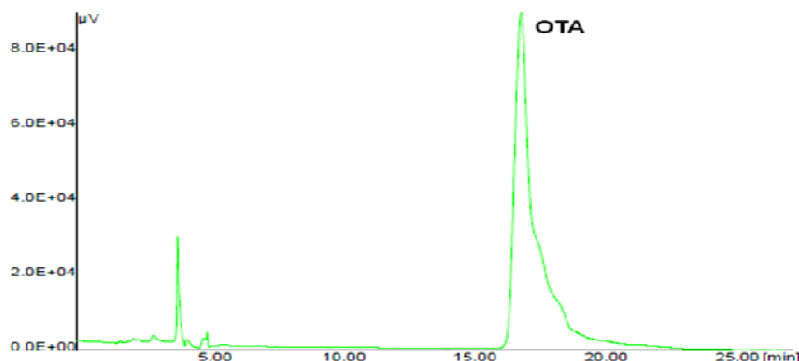


Figure 2B
HPLC Chromatogram of Ochratoxin A from contaminated sample.

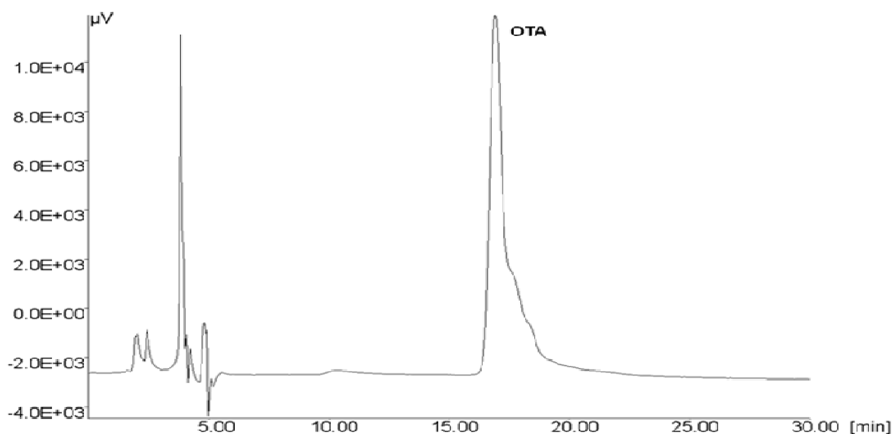


Table 4
Chemical analysis of OTA by HPTLC.

S No	Sample type	Number	% of positives	Mean concentrations of toxin (µg/kg)
1	Rice	50	20	8.3
2	Maize	50	37	15.6
3	Wheat	50	30	12.2

DISCUSSION

Majority of the citizens have in recent years developed a high tended concern for food quality and safety. Mould and mycotoxin contamination in Indian food products are rarely assessed and almost never controlled. In the present study area, the cereal grains were infected by a variety of ochratoxigenic fungi the common contaminants of these species includes, *A. niger, A. ochraceus* and *P. verrucosum.* These findings were confirmed by other workers from various countries as well.[21,22] The results dealing with frequency, density, abundance and Importance Value Index (IVI) of ochratoxigenic species confirmed that Andhra Pradesh, Tamilnadu and Karnataka fields were highly infected with ochratoxigenic producing fungi. The results of Shannon and Simpson indices showed that the

ochratoxigenic species recovered from Andhra Pradesh region were more diversified when compared with other two regions under study. However, in Tamilnadu region, ochratoxigenic species were less diversified with high dominance. Janardhana *et al*[23] reported that the maize fields of Karnataka region was highly contaminated by 24 different species of moulds belonging to 14 genera of mycotoxigenic fungi. Moreover, the present study confirmed that the earlier findings of Janardhana *et al*[23] and extended the same findings in neighbor regions Andhra Pradesh and Tamil Nadu.High mould incidences were recorded in the present study; this could be explained by the fact that there are several environmental factors like, temperature, water-activity[24], heavy rains[25], physiological conditions of the grains [26] and storage structure [27] that promote the growth of fungi in maize. *A. ochraceus, P. verrucosum* and *P. norodicum* were the potential OTA producing fungi of the region under study. Moreover, Tamilnadu region was noticed with high frequency (23%) of the OTA producing fungi than other two regions of the study. We assumed that the OTA producing fungi in Indian maize fields were affecting after crop ripening stage and the sources of contaminants were the soil, equipments and the drying yard surfaces.

The primers described in this work have been designed on the basis of ample pks sequence comparisons of several strains of *Aspergillus* species and taking into account the BLAST analyses. The specificity of the assays was tested on a number strains of *Aspergillus* and *Penicillium* species as well as on other fungi commonly associated with cereals or coffee, such as *Aspergillus, Penicillium*, *Cladosporium* or *Alternaria* specie s.The strains were obtained from Collections or isolated from the main commodities colonized by OTA-producing *Aspergillus* and *Penicillium* strains. The PCR assay developed for this toxin chemotypes might facilitate its recognition, contributing to determining its presence, distribution and relative importance in diverse substrates. The PCR assays described in this work represent an advantage in terms of time of analysis and specificity in comparison with the conventional methods of identification and the more laborious molecular methods based on AFLP and RFLP profiles. The prevalence of OTA in maize was higher than rice and wheat samples were analyzed, compared to occurrence and a mean level of OTA of 12.6 µg/kg in this study (Table-4).The occurrence of OTA in wheat samples obtained in the present study was similar to that in previous studies in other countries.[28,29,30] Wheat is the major food crop in India after rice, however, there were no reports were made regarding OTA contamination of wheat in India; hence the present study helps in a national mission to identify OTA occurrence in India. The prevalence of OTA in maize was higher than rice and wheat samples were analyzed, compared to occurrence of 50 % and a mean level of OTA of 13.6 µg/kg in this study (Table-4). Low moisture content of the soil, high day time maximum temperatures, high night-time minimum temperatures, and nutrient deficiency could be stress factors that lead to OTA production in maize.[31] Therefore, good management for cereal storage after harvest is recommended for prevention of OTA production in temperate regions like India. Maize is the world-wide major food crop in food industry, however, in India; the usage is less when compared with European countries, even though nowadays the impact of global marketing increases the corn production as well as consumption in India. So, the present study will be useful to make check OTA contamination in this upcoming crop.

In the present study a low incidence of OTA contamination of rice has been reported compared to the several previous studies worldwide.[28,30] Rice is the major staple food crop in India, especially Southern parts of India, the ochratoxigenic fungal contamination was less in rice at present conditions of farming. But, the changing styles of farming should have some precautionary measurements. Hence the developed method will be highly useful for the sensitive and specific detection of OTA in rice grains intended for consumption. The results suggested that cereals being consumed currently present no hazard to the population of Southern India. However, as a precautionary measurement one should have adopted the detection system to detect and

monitor the presence of OTA. Further research with a larger sample size and various other cereal crops needs to be conducted to determine the full extent of OTA contamination in the food chain of India.

CONCLUSION

Concern the effectiveness of ochratoxingenic fungi and OTA on humans, livestock and occurrence of these toxigenic fungi on crops of South Indian cereal grains were interlinked with ochratoxigenic moulds. Higher diversity indices of the ochratoxigenic species showed that the contamination was higher range in the studied region. Since, all the studied sites belong to temperate regions of the India, the environmental conditions especially temperature and relative humidity may be responsible for high level of OTA. In conclusion, the results of the present study reveal that there is a need for mycotoxin awareness creation among the farmers in India.

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