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RESEARCH ARTICLE

Isolation and Identification of Deleterious Fungi Associated with Stored Grains and Cattle Feedstuff of Potohar Region of Pakistan

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ABSTRACT

The growth of fungus in grains and feed is favored by improper humidity and temperature during storage contributing to loss of grain quality, infections among animals and humans and production of mycotoxins. Therefore, the current study was aimed to isolate and identify fungal species among stored grains and feedstuff of Potohar region of Pakistan. For fungal screening, ten different samples were collected from storage houses situated in different cities. These samples included wheat and corn grains from Taxila, Gujar Khan and Chakwal cities, while cattle feed samples were collected from Attock city. The investigation confirmed the presence of Rhizopus arrhizus in wheat from two different localities in Taxila and cattle feed, respectively, Aspergillus foetidus and Achaetomium globosum in wheat from Gujar Khan and Taxila, and Mucor indicus in maize from Chakwal. The most predominant fungal species was Rhizopus arrhizus. Here we are reporting the prevalence of pathogenic and toxigenic fungal species in stored grains and cattle feed of Potohar region for the first time. Inadequate storage conditions can lead to uncontrolled multiplication of fungus, so this study will assist in optimizing the storage conditions to curb its growth for assurance of healthy food for humans and animals.

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INTRODUCTION

The grains contain microbes from field at the time of harvest (Woloshuk and Martinez, 2012). Grain is a living commodity which respires under controlled storage conditions. Upon respiration, the grains produce moisture and heat raising the humidity and temperature which accelerate the fungal growth (Mohaptra *et al.*, 2016). The contaminated grains remain safe from the menace of fungal growth and production of mycotoxins if the storage conditions are well maintained. For optimization of storage conditions, it is necessary to identify the type of fungi specific to a particular area to control their growth during storage. The cattle feed, which is composed of various cereal grains, molasses, sunflower cake, mineral mixture etc. is a high source of energy and protein with high digestibility. In Pakistan, it is composed of 15-20 % wheat and 40-50 % maize grains along with other ingredients (Iqbal *et al.*, 2015). It can be contaminated with pathogenic fungi or mycotoxins if prepared with contaminated ingredients. Its aflatoxin contamination is attributed to its composition and poor storage conditions which may lead to different diseases in humans like cancer. Pathogenic fungal spores and its toxins become a part of food chain causing health hazards when contaminated meat and milk is utilized (Umer *et al.*, 2017).

According to Alconada and Moure (2022), the most frequent genera causing grain infections are Alternaria, Aspergillus, Fusarium and Penicillium. Other than these genera infecting grains, Chaetomium, Cladosporium and Rhizopus were also reported from wheat in Pakistan (Fakhrunnisa and Ghaffar, 2006) while Niaz et al. (2011) isolated Aspergillus, Fusarium, Alternaria and Absidia spp. from stored maize grains which is the second major crop grown in Pakistan after wheat. Rhizopus arrhizus (syn. R. oryzae) is an opportunistic pathogen causing mucormycoses worldwide (Dolatabadi et al., 2014) along with gastrointestinal diseases (Ribes et al., 2000), and is life threatening in patients suffered from diabetic ketoacidosis. It deteriorates the plants by producing carbohydrate digestion enzymes (Ghosh and Ray, 2011) thereby reducing the grain quality in storage. In animals, it is also associated with mycotic infections in bovine abortion cases (Knudtson and Kirkbride, 1992).

Black *Aspergilli* is a group of fungi known for spoiling common foods. Few species of this group, including *Aspergillus foetidus* are ochratoxin A (OTA) producers (Téren *et al.*, 1996) and typically contaminate the cereals (Cabañes and Bragulat, 2018). OTA is a stable compound which withstands ordinary food processing conditions. It is toxic and causes renal tumors in various animal species (Bui-Klimke and Wu, 2015) and in humans upon digestion and even inhalation (Hope and Hope, 2012).

For the first time, a genus Achaetomium and a species Achaetomium globosum were described by Rai et al. (1964). The genus is usually isolated from soil (Pote et al., 2018). Its closest genus is Chaetomium (Rodríguez et al., 2004). The difference between the two genera is based on the production of ascospores of dark chocolate brown and pale to mid brown color in Achaetomium and Chaetomium, respectively (Cannon, 1986). Chaemotium globosum is not only involved in developing human infections (Serena et al., 2003) but also produce five different types of chaetoglobosin, a mycotoxin (Li et al., 2014) but data lacks the infection causing ability and production of any type of mycotoxin by Achaemotium globosum. Few Mucor species are known to cause mycosis by invading animal and human tissues specifically among immunocompromised patients (Morin-Sardin et al., 2017) such as Mucor indicus (Chayakulkeeree et al., 2006) which also causes gastrointestinal mycosis (Deja et al., 2006).

Potohar plateau is recognized by highly variable rainfall frequency and its distribution pattern (Rashid and Rasool, 2011) which is not yet explored for the pathogenic and toxigenic fungal diversity among the stored grains and feed stuff. Therefore, the present study was aimed to investigate the occurrence of pathogenic and toxigenic fungi in the wheat and maize grains, and cattle feed under the storage facilities of Potohar region.

MATERIALS AND METHODS

Study area: The Pothohar Plateau, also known as the Potwar Plateau, is in the northern part of Pakistan. The approximate coordinates of the central part of the Pothohar Plateau are latitude approximately 32.5° to 34.0° north and Longitude approximately 72.5° to 74.5° east.

This region includes cities like Rawalpindi, Islamabad (partly), Jhelum, and Chakwal. The region experiences a climate that varies significantly between seasons due to its subtropical location. The temperature ranges between 4° C to 40° C with an annual rainfall average between 500 mm to 1000 mm, varying across different parts of the region.

Sample collection: Ten different samples were collected from different storage facilities located in different regions of Potohar area. Six wheat grain samples were collected from Gujar Khan, Chakwal, Attock and three different localities of Taxila, two samples of maize grains from Chakwal and Attock, and two samples of cattle feed from Attock and Chakwal. Each sample was collected in a sterile bag and kept in laboratory at room temperature till fungal isolation.

Isolation of fungus: The fugus was isolated by agar plate method (Panchal and Dhale, 2011). Four grains from each sample were picked aspetically, plated on Sabouraud Dextrose Agar (SDA). Similarly, the cattle feed was placed on SDA plate in four portions and the plates were incubated at room temperature untill the fungal hyphae started to develop. Each fungal colony obtained was further purified by subculturing on the separate SDA plates and incubated at room temperature for 5 days. The fungal plates were preserved at 4° C till further characterization. Six different types of fungus were randomly selected for further characterization.

DNA extraction: Fungal genomic DNA was extracted by modified CTAB method (Zhang et al., 2010). Six purified fungi were randomly selected for identification, so fresh culture of fungus was harvested from SDA plate and was ground in 500 µL lysis buffer (1 M Tris-HCl, pH 8.0; 0.5 M EDTA; 6 M NaCl; 2% CTAB). The suspension was incubated at 95°C for the first day after adding 2-3µL marcaptoethanol, 20µL proteinase K and 40 µL of 10% SDS. On day 2, DNA was purified by adding chloroform: isoamyl alcohol (24: 1) into suspension and centrifuged at 1000 rpm for 10 minutes. The upper aqueous layer was transferred into a new Eppendorf tube followed by DNA precipitation with the addition of 500µL ice chilled isopropanol. The DNA pellet was washed with 70% ethanol, air dried and re-suspended in 50µL low TE buffer (10 mM Tris-HCl; 0.1 mM EDTA, pH 8.0) and stored at -20°C. The presence of DNA was visualized on 1% agarose gel containing ethidium bromide under trans UV.

Internal transcribed spacer (ITS) gene amplification: A set of primers ITS1 (5'-CGTCACACGTTCTT CAACC-3') and ITS4 (5'-CGTTTCACGCTTCTCCG-3') (White *et al.*, 1990) were used to amplify approximately 530 bp of the ITS region from the fungal DNA extracted from six fungi. For gene amplification, 25μ L of reaction mixture, containing 12.5μ L of PCR master mixture (Abclonal, USA), 2μ L of each primer, 5.5μ L PCR water and 5μ L template DNA was prepared. A PCR of 30 cycles was performed at the following conditions: initial denaturation at 95°C for 5 min, denaturation at 95°C for 30 sec, annealing at 57°C for 35 sec, extension at 72°C for 40 sec followed by final extension at 72°C for 5 min. The amplified PCR products were run on 1.5% agarose gel to confirm the size of a product. The amplified products were sent to Macrogen, Korea for their sequencing. The sequenced ITS regions were aligned with the nucleotide sequences retrieved from the NCBI database for maximum homology by using BLAST. The ITS nucleotide sequences of each fungus were submitted to GenBank for accession numbers.

Analysis of nucleotide sequences of Pakistani fungal strains: Nucleotide sequences of Pakistani fungal strains were compared to reference strains retrieved from GenBank for each fungal species. Genbank accession numbers DQ641279, NR_163668, NR_077173 and NR_157458 were used as reference for *Rhizopus arrhizus*, *Aspergillus foetidus*, *Mucor indicus*, and *Achaetomium globosum*, respectively. The nucleotide sequences were aligned and edited by Geneious® Version 6.1.8.

Phylogenetic analysis of fungal isolates: To construct a phylogenetic tree by neighbor-joining method (Saitou and Nei, 1987), the closely related sequences to our fungal isolates were retrieved from NCBI and were aligned by Clustal W program, followed by the phylogenetic tree construction by Mega X software (Kumar *et al.*, 2018).

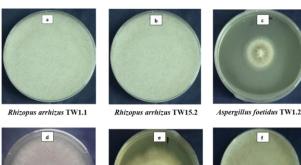
RESULTS

Identification of fungal isolates: Six fungal species (Fig. 1) were isolated from grain and cattle feed storage facilities located in different cities i.e., Taxila, Gujar khan, Chakwal and Attock of Potohar region. Among all the fungal species, five were isolated from the stored grains while one was isolated from the cattle feed. These are likely to be field fungi which invade grains during harvesting of cereal crops. They were identified at species level by amplifying their ITS gene (Fig. 2) followed by sequencing. The genetic homology of fungal isolates TW1.1, TW15.2, TW1.2, GW3.2, CC12.1 and AW11 was found 100, 99.8, 100, 99.82, 98.70 and 100% with Rhizopus arrhizus, Rhizopus arrhizus, Aspergillus foetidus, Achaetomium globosum, Mucor indicus and Rhizopus arrhizus, respectively. The accession numbers assigned by GenBank are shown in Table 1.

Comparison of nucleotide sequences of Pakistani fungal strains with reference strains: Comparison of *Achaetomium globosum* GW 3.2 (OP948735) with the reference strain (GenBank accession no. NR_157458) revealed that there was only one difference in the nucleotide sequences which was a deletion at nucleotide position 82. *Aspergillus foetidus* TW1.2 (OP94873) comparison with reference strain (GenBank accession no. NR_163668) revealed that both are almost identical strains at least over the region sequenced in this study.

 Table I: Fungal species, their source, origin and GenBank accession numbers

Fungal Species	Source	Origin	Accession numbers
Rhizopus arrhizus TW1.1	Wheat	Taxilla	OP948736
Rhizopus arrhizus, TW15.2	Wheat	Taxilla	OP948739
Aspergillus foetidus TW1.2	Wheat	Taxilla	OP948737
Achaetomium globosum GW3.2	Wheat	Gujar Khan	OP948735
Mucor indicus CC12.1	Corn	Chakwal	OP948738
Rhizopus arrhizus AVVI I	Feed Concentrate	Attock	OP948734





Achaetomium globosum GW3.2

Rhizopus arrhizus AW11

Fig. 1: Colony morphology of (a) *Rhizopus arrhizus* TW1.1, (b) *Rhizopus arrhizus*, TW15.2, (c) *Aspergillus foetidus* TW1.2, (d) *Achaetomium globosum* GW3.2, (e) *Mucor indicus* CC12.1 and (f) *Rhizopus arrhizus* AW11

Mucor indicus CC12.1

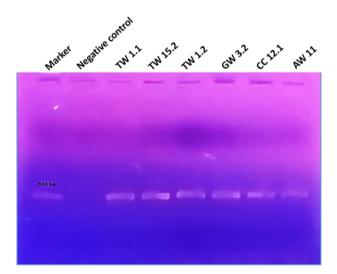


Fig. 2: PCR amplification of ITS region (~ 530 bp) of fungal species isolated in current study. The ITS of *Rhizopus arrhizus* TW1.1, *Rhizopus arrhizus* TW15.2, *Aspergillus foetidus* TW1.2, *Achaetomium globosum* GW3.2, *Mucor indicus* CC12.1 and *Rhizopus arrhizus* AW11 were amplified by PCR and visualized on 1.5 % agarose gel. Marker is the known PCR product of ~ 530 bp from a previously confirmed PCR using ITS1 and ITS4 primers.

Multiple mutations including substitution, insertion and deletion were identified when *Rhizopus arrhizus* AW11, *Rhizopus arrhizus* TW1.1 and *Rhizopus arrhizus* TW15.2 sequences were compared with the reference strain (GenBank accession no. DQ641279). A total of 6 substitutions and 2 deletions were identified in the sequence of *Mucor indicus* CC12.1 (OP948738) when compared with the reference strain (GenBank accession no. NR_077173). Nucleotide variations are shown in Fig. 3, 4, 5 and 6, respectively.

Phylogenetic analysis based on ITS region nucleotide sequences: A phylogeny was constructed to study the evolutionary relationship of isolated fungal species with each other and other fungus of the same genera (Fig. 7). *Saccharomyces cerevisiae* was used as an outgroup and the phylogenetic tree was rooted on it. The phylogenetic tree shows two distinct groups. One group is comprised of *Chaetomium/ Achaetomium* and *Aspergillus* species while

Fig. 3: Achaetomium globosum (OP948735) nucleotide alignment with the reference strain (NR_157458).

Fig. 4: Aspergillus foetidus (OP948737) nucleotide alignment with the reference strain (NR_163668).

Fig. 5: Rhizopus arrhizus (OP948734, OP948736, OP948739) nucleotide alignment with the reference strain (DQ641279).

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2. Aspergillus foetidus TW1.2 (DP948737) 3. Aspergillus foetidus PPMO2 (MZ955454) 1. Aspergillus foetidus CBS 121.28 (NR_163668) 2. Aspergillus foetidus TW1.2 (DP948737) 3. Aspergillus foetidus CBS 121.28 (NR_163668) 2. Aspergillus foetidus CBS 121.28 (NR_163668) 3. Aspergillus foetidus CBS 121.28 (NR_163668) 1. Aspergillus foetidus CBS 121.28 (NR_163668) 4. Aspergillus foetidus CBS 121.28 (NR_163668) 2. Aspergillus foetidus CBS 121.28 (NR_163668) 2. Aspergillus foetidus CBS 121.28 (NR_163668) 4. Aspergillus foetidus CBS 121.28 (NR_163668) 4. Aspergillus foetidus CBS 121.28 (NR_163668) 4. Aspergillus foetidus CBS 121.28 (NR_16367) 4. Aspergillus foetidus CBS 121.28 (NR_16368) 4. Aspergillus foetidus 4.	CATCCG CATCCG GGGGGGG GGGGGGG 170 GTCTGA GTCTGA GTCTGA GTCTGA 23 TGGATC	TGTCT TGTCT 120 CGCCT CGCCT CGCCT AAGCG AAGCG AAGCG CTCTTG TCTTG	A TTGT A TTGT C TGCC C TGCC C TGCC TGCAG TGCAG GTTCC GTTCC	ACCCT ACCCCG 130 CCCCGG CCCCGG CCCCGG TCTGAG TCTGAG GGCAT GGCAT	GTTGC GGCCC GGCCC GGCCC GGCCC GGTTGA GTTGA 2% CGATG CGATG	TTCGG TTCGG GTGCC GTGCC GTGCC TTGAA TTGAA TTGAA AAGAA	CGGGGC CGGCCGC CGCCGC CGCCGC CGCCGC TGCAA TGCAA TGCAA TGCAA 200 CGCAGC CGCAGC	CGCCG CGCCG AGACCC AGACCC AGACCC 210 CAGTT CAGTT CAGTT CAGTT CAGTT	CTTGTC CTTGTC CTTGTC CAACA CCAACA CCAACA AAAACT AAAACT 270 CGATA SCGATA	CGGCCGCC CGGCCGCC CGGCCGCC CGAACAC CGAACAC 220 TTCAACA TTCAACA TTCAACA
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2. Aspergillus foetidus TVH.2 (OP948737) 3. Aspergillus foetidus TVH.2 (OP948737) 1. Aspergillus foetidus CBS 121.28 (NZ_163668) 2. Aspergillus foetidus CBS 121.28 (NZ_163668) 3. Aspergillus foetidus PPMC2 (M2955454) 1. Aspergillus foetidus CBS 121.28 (NZ_163668) 2. Aspergillus foetidus TVH.2 (OP948737) 3. Aspergillus foetidus CBS 121.28 (NZ_163668) 2. Aspergillus foetidus CBS 121.28 (NZ_163668) 2. Aspergillus foetidus CBS 121.28 (NZ_163668) 3. Aspergillus foetidus CBS 121.28 (NZ_163668) 3. Aspergillus foetidus CBS 121.28 (NZ_163668) 4. Aspergillus foetidus CBS 121.28 (NZ_163668) 5. Aspergillus foetidus CBS 121.28 (NZ_16368) 5. Aspergillus foetidus CBS 121.28 (N	CATCCG CATCCG GGGGGG GGGGGGG 170 GTCTGA GTCTGA GTCTGA GTCTGA TGGATC TGGATC GAATTG GAATTG	TGTCT TGTCT 120 CGCCT CGCCT CGCCT AAAGCG AAAGCG AAAGCG TCTTG TCTTG TCTTG 220 CAGAA	ATTGT ATTGT CTGCC CTGCC CTGCC CTGCC GTGCAG GTTCC GTTCC GTTCC GTTCC GTTCCAG GTTCCAG	ACCCT 130 CCCCG CCCCG CCCCG CCCCG TTTTTTTTTT	GTTGC GGCCC GGCCCC GGCCCC GGCCCC GGCCCC GGTTGA GTTGA GTTGA GTTGA GTTGA GTTGA GTTGA GTTGA CATCG CATCG CATCG CATCG	TTCGG TTCGG GTGCC GTGCC GTGCC TTGAA TTGAA TTGAA ATTGAA AAGAA AAGAA AAGAA AAGAA AAGAA AAGAA	CGGGCC CGCCGC CGCCGC CGCCGC CGCCGC TGCAA TGCAA TGCAA CGCAG CGCAG CGCAG CGCAG CGCAG CGCAG CGCAG CGCAG CGCAG CGCAG CGCAG CGCAG CGCAG CGCAG CGCAG CGCAG CGCAG CGCAG CGCAG CGCAG CGCAG CGCCGC CGCCGC CGCCGC CGCCGC CGCCGC CGCCGC	CGCCCG CGCCCG GGACCCG AGACCC AGACCC 210 CAGTC CAGTC CAGTC CAGTC CGAAAT CGAAAT 320 CGCACA	CAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA AAAAC CCAACA AAAAC CCAACA AAAAC CCAACA CCAACA CCAACA CCAACA AAAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCAACA CCACACA CCACACA CCACACA CCACACA CCACACA CCACACA CCACACA CCACACA CCACACA CCACACA CCACACA CCACACA CCACACA CCACACA CCACACA CCACACA CCACACACA CCACACA CCACACA CCACACACA CCACACA CCACACACACACA CCACACACACACA CCCACACACACACACACACACACACACACACACACACACA	
2. Aspergillus foetidus TW1.2 (DP948737) 3. Aspergillus foetidus PPMO2 (MZ955454) 1. Aspergillus foetidus CBS 121.28 (NR_163668) 2. Aspergillus foetidus TW1.2 (DP948737) 3. Aspergillus foetidus PPMO2 (MZ955454) 1. Aspergillus foetidus CBS 121.28 (NR_163668) 2. Aspergillus foetidus CBS 121.28 (NR_163668) 2. Aspergillus foetidus CBS 121.28 (NR_163668) 3. Aspergillus foetidus CBS 121.28 (NR_163668) 4. Aspergillus foetidus CBS 121.28 (NR_163668)	CATCCG CATCCG GGGGCG GGGGCG GGGGGG GGGGGG GGGGGG GGATC TGGATC TGGATC GAATTG GAATTG GAATTC GAATTCCG AATTCCG	TGTCT TGTCT 120 CGCCT CGCCT CGCCT CGCCT CGCCT CGCCT CGCCT CGCCT CGCCT CCCCC CCCCC CCCCC CCCCC CCCCC CCCCC CCCC	ATTGT ATTGT CTGCC CTGCC CTGCC TGCAG TGCAG GTTCCAG GTTCCAG GTTCCAG TTCAG TTCAG CATCC CATCC		GTTGC GTTGC GCCCC GCCCC GTTGA GTTGA GTTGA GTTGA GTTGA GTTGA CATCG CATCG CATCG CATCG CATCG CATCG CATCG CATCG CATCG CATCG CCATCG CGAGC	TTCGG GTGCC GTGCC GTGCC GTGCC GTGCC TTGAA TTGAA TTGAA TTGAA AAGAA AAGAA AAGAA AAGAA AGTCT GTCAT GTCAT	CGGGCCGC CGCCGCCGC CGCCGCCGC CGCCGCGC CGCCGC	CCCCCG CGCCG CGCCG CGCCG CGCCG AGACC CAGAC CCGCG CAGTT CCAGTT CCAGTT CCAGTT CCAGTT CCAGTT CCAGTT CGAAAT CGAAAT CGAAAT CGAAAT CGCACA CGCACA CGCACA CGCCCA		GGCCGCC GGCCGCC CGAACAC CGAACAC CGAACAC CGAACAC CGAACAC TTCAACP TTCAACP TTCAACP TTCAACP CCCCGGCCGC CCCCTGC CCCCTGC CCCCTGC CCCCTGC GGCTTGTC GGCTTGTC
 Aspergillus foetidus TW1.2 (CP48737) Aspergillus foetidus PPMO2 (M295454) Aspergillus foetidus PPMO2 (M295454) Aspergillus foetidus CBS 121.28 (NR_163668) 	CATCCG CATCCG GGGCGG GGGCGGG 170 GGCCGGGGGG 170 GTCTGA GTCTGA GTCTGA GTCTGA GTCTGA GTCTGA GAATTG GAATTG GAATTG GAATTCCG AATTCCG GGGGGGGGGG		ATTGT ATTGT CTGCC CTGCC CTGCC CTGCC TGCAG TGCAG TGCAG GTTCC GTTCCAG TTCAG TTCAG 350 CATGC CATGC CATGC		GTTGC GGCCC GGCCC GGCCC GGCCC GGCCC GGCCC GGCCC GGCCC GGCCC GGATG GATGG GATGG GAGC GAGC	TTCGG TTCGG GTGCCG GTGCCG GTGCCG TTGAA TTGAA TTGAA TTGAA AAGAA AAGAA AAGAA AAGAA AAGAA AGTCT GTCAT 420 GGGAC	C G G G C C G C C G C C G C C G C C G C C G C C G C C G C C G C C G C C G C C G C A C C G C A C C G C A C C G C A G C G C	CCCCCG CGCCCG T ¹⁰ AGACCC AGACCC AGACCC AGACCC CGCGCT CGGCT CGAAAT CGAAAT CGAAAT CCCCCCA CCCCCA CCCCCA CCCCCA CCCCCA CCCCCA CCCCCA CCCCCA		
2. Aspergillus foetidus TVH 2 (OP948737) 3. Aspergillus foetidus TVH 2 (OP948737) 3. Aspergillus foetidus CBS 121.28 (NZ_163668) 2. Aspergillus foetidus CBS 121.28 (NZ_163668) 3. Aspergillus foetidus TVH 2 (OP948737) 3. Aspergillus foetidus CBS 121.28 (NR_163668) 2. Aspergillus foetidus CBS 121.28 (NR_163668) 3. Aspergillus foetidus CBS 121.28 (NR_163668) 4. Aspergillus foetidus CBS 121.28 (NR_163668) 3. Aspergillus foetidus CBS 121.28 (NR_163668) 4. Aspergillus foetidus CBS 121.28 (NR_163668) 5. Aspergillus foetidus CBS 121.28 (N	CATCCG CATCCG GGGCGG GGGCGGG 170 GGCCGGGGGG 170 GTCTGA GTCTGA GTCTGA GTCTGA GTCTGA GTCTGA GAATTG GAATTG GAATTG GAATTCCG AATTCCG GGGGGGGGGG		A TIGI A TIGI CIGCC CIGCC CIGCC CIGCC CIGCAG TICAG GIICC GIICC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAICCC GICCC	ACCCT 170 CCCCG CCCCG CCCCG CCCCG CCCCG CCCCG CCCCG CCCCG CCCCG CCCGG CCCGG CCCGG CCCGG CCCGG CCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCCGG CCCGG CCCGG CCCGG CCCGG CCGG CCGG CCGG CCGG CCGG CCGG CCGG CCGG CCGG CCGG CCGG CCGG CCGG CCGG CCGG CCGG CCGG CCGG CCGG CCGG CCGG CCGGC CCGGC CCCG CCGCG CCCG CCGCG CCCCG CCCGCG CCCCG CCCGCG CCCGCG CCCCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCGCG CCCGCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCG CCCGCGCG CCC	GATGGAGG GGAGGCCC GGCCCC GGCCCC GGCCCC GGCCCC GGCCCC GGCCCC GGAGGCCCC GATGGAGC CGAGGCCCGAGC CCGAGCCCCGGGGCCCCGGGGC	TTCGG TTCGG GTGCCG GTGCCG GTGCCG TTGAA TTGAA TTGAA TTGAA AAGAA AAGAA AAGAA AAGAA AGTCT GTCAT GTCAT GTCAT GCGAC GGGAC	C G G G C C G G G C C G G G G C C G C G	CGCCGG TSO CGCCGG SAGACC SAGACC SAGACC CAGT CAGTI CAGTI CAGTI CAGTI CAAAT SCAAAT SCAAAT SCAAAT CGAAAT CGAAAT CGAAAT CGAAAT CGAAAT		
Aspergillus foetidus TW1.2 (OP948737) Aspergillus foetidus PPMO2 (M295454) Aspergillus foetidus PPMO2 (M295454) Aspergillus foetidus CBS 121.28 (NR_163668) Aspergillus foetidus PPMO2 (M2955454) Aspergillus foetidus PPMO2 (M295454) Aspergillus foetidus PPMO2 (M295454) Aspergillus foetidus PPMO2 (M295454) Aspergillus foetidus PPMO2 (M2954545) Aspergillus foetidus PPMO2 (M2	CATCCG CATCCG GGGGCG GGGGCG GGGGCG GGGGGGG GGGGGGG		A TIGI A TIGI CIGCC CCTCCCC TGCAG TGCAG GITCC GITCCAG CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC CAIGC		G T T G C G C C C G A T G G A T C G A T C G A T C G C A T C G C A T C G C A T C G C C C G A G C C C A T C G C C C G A G C C C C C G G C C C C G G G C C C C G G G C C C C	TTCGG TCGG GTGCC GTGCC GTGCC GTGCC TTGAA TTGAA TTGAA TTGAA TTGAA AGAA A	CGCACCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC	CGCCGG 150 CGCCGG 3GGACGC 3GGACCC 3GGACCC 3GGACCC CGGCGGT CGGGTT CGGTT CGGAAAT CGAAAT CGCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACA CCCCCACACA CCCCCACACA CCCCCCACACA CCCCCACACACA		

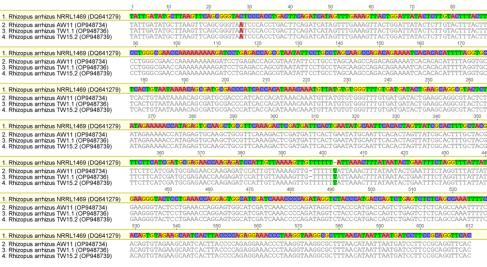
1. Aspergillus foetidus CBS 121.28 (NR_163668) 2. Aspergillus foetidus TW1.2 (OP948737) 3. Aspergillus foetidus PPMO2 (MZ955454) GCCT

GCCGACG

ACCCGCTGAACTTAAGCA

ACCCGC TGAAC TTAAGCA ACCCGC TGAAC TTAAGCA

1. Aspergillus foetidus CBS 121.28 (NR_163668) 2. Aspergillus foetidus TW1.2 (OP948737) 3. Aspergillus foetidus PPMO2 (MZ955454)



TCCAACCA

GCCTGCCGACGTTTTCCAACCATTCTTTCCAGGTTGACCTCGGATCAGGTAGGGAT GCCTGCCGACGTTTTCCAACCATTCTTTCCAGGTTGACCTCGGATCAGGTAGGGAT 570 578

Fig. 6: Mucor indicus (OP948738) nucleotide alignment with the reference strain (NR 077173).

Fig. 7: A phylogenetic tree was

sequences of fungal species isolated

in this study and related sequences

fungal isolates sequenced in current

retrieved from NCBI. Pakistani

study are indicated as black dots

and the reference sequences for

squares. Saccharomyces cerevisiae

was used as an outgroup and the

tree is rooted on Saccharomyces

cerevisiae. Bootstrap percentages

are indicated below the nodes.

Branch lengths are highlighted

above the branches. Bootstrap

value of 1000 was adjusted.

Evolutionary analyses were

conducted in MEGAII.

each species are indicated by

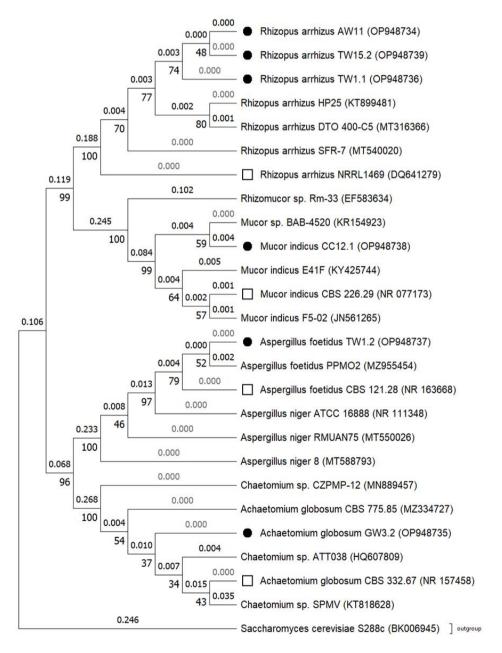
inferred by Neighbor-joining

method using combined ITS

	1	10	20	30	40	50	60	70
1. Mucor indicus CBS 226.29 (NR_077173)	GGTGAACCT	GCGGAAGG	ATCATTAAA	TAATCTGAT	AA <mark>TTC</mark> AA <mark>T</mark> AA	TTATCTTATTT	ACTGTGAAC	TGTTTTA
2. Mucor indicus CC12.1 (OP948738) 3. Mucor indicus F5-02 (JN561265)	GGTGAACCT GGTGAACCT 80	GCGGAAGG. GCGGAAGG. 90	АТСАТТААА АТСАТТААА	TAATCTGAT TAATCTGAT	AATT <mark>A</mark> AATAA AATTCAATAA 110		ACTGTGAAC ACTGTGAAC 130	TGTTTTTA TGTTTTTA 140
1. Mucor indicus CBS 226.29 (NR_077173)	TTTATGACG	TATAAGGG	GATGTCTT	AGGCTATAA	GGGTAGGCCT	A <mark>T</mark> GGAA <mark>T</mark> G TT A	ACCTAGTCA	TAGTCAAG
2. Mucor indicus CC12.1 (OP948738) 3. Mucor indicus F5-02 (JN561265)	TTTATGACG TTTATGACG 150	AATAAGGG TATAAGGG 160	GATGTCTTT GATGTCTTT 170	AGGCTATAA AGGCTATAA 180	GGGTAGGCCT GGGTAGGCCT 190	ATGGAATGCTA ATGGAATGTTA 200		TAGTCAAG TAGTCAAG
1. Mucor indicus CBS 226.29 (NR_077173)	CTTGATGCT	TGGTACCC	SATTATTAC	TTACCAAAA	GAA <mark>TTC</mark> AG TT	TAAAATATTGT	AACATAGAC	CTAAAAAA
2. Mucor indicus CC12.1 (OP948738) 3. Mucor indicus F5-02 (JN561265)	CTTGATGCT CTTGATGCT 220	TGGTACCC TGGTACCC 230	GATTATTAC GATTATTAC 240	TTACCAAAA TTACCAAAA 250			AACATAGAC AACATAGAC 280	СТАААААА СТАААААА 290
1. Mucor indicus CBS 226.29 (NR_077173)	TCTATAAAA	CAACTTTT	AACAA <mark>T</mark> GGA	TCTCTTGGT	TCTCGCATCG	ATGAAGAACGT	AGCAAAGTG	CGATAACT
2. Mucor indicus CC12.1 (OP948738) 3. Mucor indicus F5-02 (JN561265)	TCTATAAAA TCTATAAAA 300		AACAATGGA AACAATGGA 10	TCTCTTGGT TCTCTTGGT 320	TCTCGCATCG TCTCGCATCG 330	ATGAAGAACGT ATGAAGAACGT 340	AGCAAAGTG AGCAAAGTG 350	CGATAACT CGATAACT 360
1. Mucor indicus CBS 226.29 (NR_077173)	AGTGTGAAT	TGCATATT	CAGTGAATC	ATCGAGTCT	TTGAACGCAT	CTTGCACTCAA	TGGTATTCC	ATTGAGTA
2. Mucor indicus CC12.1 (OP948738) 3. Mucor indicus F5-02 (JN561265)	AGTGTGAAT AGTGTGAAT 370	TGCATATT TGCATATT 380	CAGTGAATC CAGTGAATC 390	ATCGAGTCT ATCGAGTCT 400	TTGAACGCAT	CTTGCACTCAA	TGGTATTCC TGGTATTCC 430	ATTGAGTA
1. Mucor indicus CBS 226.29 (NR 077173)	C <mark>GCCTGTTT</mark>	CAGTATCA.	AAAACAACC	CTTATTCAA	AATTCTTTT	TTGAATAGATA	TGAG TG TAG	CAACCTTA
2. Mucor indicus CC12.1 (OP948738) 3. Mucor indicus F5-02 (JN561265)	CGCCTGTTT CGCCTGTTT 440	CAGTATCA CAGTATCA 450	AAAACAICC AAAACAACC 460	CTTATTCAA CTTATTCAA 470	A <mark>GTT - TTTTT</mark> AATTCTTTTT 480	TTGAATAGATA TTGAATAGATA 490	TGAGTGTAG TGAGTGTAG 500	
1. Mucor indicus CBS 226.29 (NR_077173)	CAAG TTGAG	ACATTTTA.	AA T AAAG T C	AGGCCATAT	CG TGG A T TG A	GTGCCGATACT	TTTTTTTTTT	TTGAAAAG
2. Mucor indicus CC12.1 (OP948738) 3. Mucor indicus F5-02 (JN561265)	CAAGTTGAG CAAGTTGAG 52		AATAAAGTC AATAAAGTC 530	AGGCCATAT AGGCCATAT 540	CGTGGATTGA CGTGGATTGA 550	GTGCCGATACT GTGCCGATACT 560	TTTTTTAATT TTTTTTAATT 570	TTGAAAAG TTGAAAAG 580
1. Mucor indicus CBS 226.29 (NR_077173)	GTAAAGCAT	GTTGATGT	CCGCTTTTT	GGGCCTCCC	AAATAACTTT	TTAAACTTGAT	CTGAAATCA	GGTGGGAT
2. Mucor indicus CC12.1 (OP948738) 3. Mucor indicus F5-02 (JN561265)	GTAAAGCAT GTAAAGCAT 590	GTTGATGT GTTGATGT 600	CTGCTTTTT CCGCTTTTT 61		AAATAACTTT AAATAACTTT	TTAAACTTGAT TTAAACTTGAT	CTGAAATCA CTGAAATCA	GGTGGGAT GGTGGGAT
1. Mucor indicus CBS 226.29 (NR_077173)	TACCCGCTG	AACTTAGA	GCATATCAA	TAAGC				

2. Mucor indicus CC12.1 (OP948738) 3. Mucor indicus F5-02 (JN561265)

TACCCGCTGAACTTA-AGCATATCAATAAGC TACCCGCTGAACTTAA-GCATATCAATAAGC



the other group consists of *Rhizopus* and *Mucor* species. Chaetomium spp. form the basal clade including Achaetomium globosum GW3.2, depicting a common

ancestor for other isolated fungal species. The clade of Aspergillus spp. is originated from the basal cluster. Aspergillus foetidus TW1.2 is more closely related to the

group consisting of *Rhizopus arrhizus* AW11, *Rhizopus arrhizus* TW1.1 and *Rhizopus arrhizus* TW15.2 than other *Aspergillus* species included in the current analysis. From this group, a cluster of *Rhizopus* spp. encompassing *Rhizopus arrhizus* AW11, *Rhizopus arrhizus* TW1.1 and *Rhizopus arrhizus* TW15.2 is diverged, and make another group which includes *Mucor* spp. *Mucor indicus* CC12.1, isolated in this study, also fall in this group.

DISCUSSION

In our study, *Rhizopus arrhizus* TW1.1 and *Rhizopus arrhizus* TW15.2 were prevalent among wheat collected from two different localities of Taxila. *Rhizopus arrhizus* prevails in Potohar region as Liaquat *et al.* (2019) isolated it from tomato where it was found responsible for causing brown rot. Arif *et al.* (2017) also reported it from the same region as a causative agent of fruit rot in yellow oleander. Various cereal grains other than wheat also inhabit *Rhizopus arrhizus* as shown by the studies of Wilson *et al.* (2016) and Cara *et al.* (2018), who isolated these fungi from stored maize and barley grains.

Another fungus in this study was identified as *Aspergillus foetidus* TW1.2 which was isolated from wheat grains collected from Taxila. The *Aspergillus* spp. was found among the fresh and the stored sesame seeds of Potohar region, capable of producing aflatoxins as investigated by Ajmal *et al.* (2022). According to Al-Wadai *et al.* (2013), *Aspergillus* is the most common occurring genera among the wheat grains, and if the wheat and other grains contaminated with *Aspergillus* are used as ingredients in animal feed, they may produce toxins which is then consumed by animals. In this regard, the study by Usman *et al.* (2019) focused on isolation of aflatoxigenic *Aspergillus* spp. from animal feed.

Along with the isolation of *Rhizopus arrhizus* and *Aspergillus foetidus*, another species *Achaetomium globosum* GW3.2 was isolated from the wheat of Gujar Khan region. *Achaetomium* spp. are soil saprophytes. Rodríguez *et al.* (2004) isolated new species of *Achaetomium* from Indian soil which supports our finding that wheat attained *Achaetomium globosum* from field and retained during storage. To our knowledge its presence among cereal grains is not reported yet. It can be considered toxigenic as *Achaetomium* originated from *Chaetomium* (Rodríguez *et al.*, 2004) which is also indicated by the phylogenetic tree constructed in this study.

The results showed that *Mucor indicus* CC 12.1 was associated with corn collected from Chakwal. *Mucor* spp. is also prevalent in Potohar region known for causing rot infection in fruits such as rotting of *Eriobotrya japonica* (Abbas *et al.*, 2018). The presence of *Mucor* spp. was also noticed among the maize grains collected from fields of Thailand (Inyawilert *et al.*, 2020) while its presence was observed among wheat grains imported from Argentina and Kazakistan stored in Iranian silos (Okhovvat and Zakeri, 2003).

To maintain the quality of feed and to preserve animal health, it is prerequisite to keep the constant check on raw materials used as ingredients (Krnjaja *et al.*, 2010). Veterinary feeds are produced mainly from wheat and maize grains (Khalifa *et al.*, 2022). The cattle feed collected in this study was contaminated with *Rhizopus* *arrhizus* AW11. It can be assumed that the fungal contamination present in cattle feed might be due to the addition of *Rhizopus oryzae* contaminated wheat grains, and its presence in wheat samples has been shown in current study. Our findings are in accordance with the results of Krnjaja *et al.* (2010) who reported *Rhizopus* as a dominant genus in animal feed. In short, the *Rhizopus arrhizus* was a dominant fungal species among all the samples collected from Potohar region.

Conclusions: Our study revealed the presence of pathogenic and toxigenic fungal species among stored wheat, maize and feed concentrate of Potohar region of Pakistan for the first time. The wheat was susceptible to pathogenic Rhizopus arrhizus, toxigenic Aspergillus foetidus and Achaetomium globosum while pathogenic Mucor indicus was isolated from maize. Rhizopus arrhizus was also found in cattle feed. If the growth of deleterious fungi is not controlled by maintaining storage conditions, it can deteriorate grain quality that may cause infections and produce toxins to affect the health of humans and animals. The novel information of this study, which revealed the types of fungi, can be considered for the optimization of storage conditions to protect the quality of wheat and maize grains and ensure the quality of feed concentrate made from cereal grains.

Authors contributions: MAK, IAK and AHT designed and executed the project and organized the data. MAS, NN and MAZ made a significant contribution to the idea of the study and interpreted the results and drafted the manuscript. SAB, RHP and YA reviewed the manuscript. SS and BJ facilitated in the research and collected and compiled the data.

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