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Development of sensitive polyclonal antibodies against dominant stored wheat grain fungus for its immunological detection

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Abstract

Fungal infestation causes deterioration of stored food grains. Most fungal species produce secondary metabolites like aflatoxins which are highly toxic to animals and humans. *Aspergillus flavus* has been found to be the predominant contaminant in stored wheat grains collected from the godowns of Food Corporation of India, West Bengal. The present study focuses on the development of sensitive polyclonal antibodies (PAbs) for molecular immunological detection of dominant toxigenic fungus. Pure *A. flavus* isolate was cultured on

coconut agar media and its spores were harvested and inactivated by 4% formaldehyde. The inactivated spores were injected into a rabbit along with Freund's complete/incomplete adjuvant for the development of PAbs. Specificity of the raised antibodies in rabbit serum was examined by enzyme-linked immunosorbent assay (ELISA) using spore proteins as antigen obtained by bead beating method. Out of several proteins (ranging from 10 to 200 kDa present in spore, only two prominent proteins of around 76 kDa and 100 kDa were detected by western blot analysis using raised polyclonal antiserum. The PAbs were purified with protein A column followed by spore proteins conjugated CNBr activated sepharose column for its use in the detection of fungal antigens. This highly purified raised antibody can be used for the development of rapid, sensitive, and accurate techniques (such as dot blot/ELISA) for the detection of toxigenic fungi present in stored wheat grains.

Keywords: Spore Protein, Polyclonal Antibody, Fungus Detection

Introduction

In India, wheat is the third most produced and consumed cereal grain. It has economic importance as a staple food all over the world. It is a seasonal crop and hence needs to be stored safely as buffer stock for year-round consumption. It is estimated that around 30% of the total grain produced in the country is supplied to government storage house like food corporation of India (FCI) for keeping as buffer stock. Sometimes improper storage and handling can cause huge economical loss. Estimated loss of staple food grains during storage due to different biotic and abiotic factors varies widely. It may account to 10% worldwide but can reach 50% in tropical regions (Magan et al., 2007). The biotic factors of storage loss mainly include fungal infestation. Fungal infestation may lead to loss of seed germination capacity, viability, decoloration, foul smell and change in nutritional value (Birck et al., 2006). The most common fungal species causing grain spoilage in storage are *Eurotium* and *Aspergillus* (Tournas et al., 2018). *Aspergillus* species like *A. flavus* are particularly important because they are able to colonise even at very low moisture content on a range of food matrices, resulting in spoilage, and produce varied group of mycotoxins which may lead to refusal of stored food grains (Sohbatzadeh et al., 2016; Aldars-García et al.,2018). *Aspergillus flavus* can also cause aspergillosis in immune-compromised individuals (Amaike and Keller, 2011). Therefore, determination of the mycological contamination of stored grains is a very important issue as it is destined to be used as food and animal feeds. During the last decade, several direct methods like dilution plating, measurement of volatile compounds, evaluation of ergosterol or chitin level and next gen sequencing (NGS), and indirect methods like randomly amplified polymorphic DNA (RAPD) analysis, amplified ribosomal DNA restriction analysis (ARDRA) and denaturing gradient gel electrophoresis (DGGE) have been developed for the detection of fungi. However, numerous disadvantages are associated with these techniques. The direct methods are time consuming, labor intensive, and require mycological expertise; and are not completely accurate (Gourama and Bullerman, 1995). In indirect methods: it is impossible to distinguish among species or to yield quantitative data (Darling and Blum, 2007). As a consequence, there is a clear and urgent need to develop a new reliable method that will be highly specific, relatively rapid, inexpensive, and replicative. Immunological and molecular techniques offer such an alternative. The immunological method is based on antigen and antibody (either monoclonal /MAb/ or polyclonal /PAb/) interaction. Different sources of fungal material like surface washes from the growth medium, mycelial homogenate and extracellular polysaccharide have been used as antigens for the production of antibody but no study has been done by directly using fungal spore. In current study, we have isolated *A. flavus* fungus from stored wheat grain, developed polyclonal antibodies by directly using inactivated *A. flavus* spore and specificity of antibodies was analysed using ELISA and western blot method.

Materials and Methods

Isolation and characterization of *A. flavus* fungus from stored wheat grain

Stored wheat grains were collected from Food Corporation of India (FCI) godown of West Bengal. The grains were plated on aspergillus differentiation agar (AFPA) media for isolation of *A. flavus*. The fungal isolate was identified by analysing morphological characteristic of colony and sequencing

internal transcribed spacer (ITS) region of ribosomal DNA gene. The ITS region was amplified using universal (ITS1 and ITS4) primer set by PCR, then cloned in pTZ57R/T vector (Fermentas Life Sciences, EU) and sequenced in DNA sequencer (ABI 3500, USA). Homology search of nucleotide sequence was performed against the Genbank database for identication of fungal species.

Spores collection and inactivation

The identified *A. flavus* isolate was cultured on coconut agar media [100ml coconut milk and 2% agar] for 7 days at 28°C. The spores were collected in 1xPBS containing 0.1% tween 80. It was then inactivated by keeping in 4% formaldehyde solution, for 5 days at 4°C and followed by several wash with PBS. The inactivated spores were cultured on PDA media for 7 days at 28°C for analysis of inactivation efficiency. The spore density was calculated on haemocytometer under microscope.

Generation of polycolonal antiserum against inactivated spore

Polyclonal antiserum against inactivated *A. flavus* spore proteins was raised by immunization of rabbit (New Zealand) using the standard 6-month antibody production protocol. 10^8 spore per injection in Freund's complete adjuvant was subcutaneously injected for the first time and then in Freund's incomplete adjuvant for subsequent injections (at an interval of four weeks). Blood was collected 10 days after every injection. Sera were harvested by centrifugation at 3000 g for 15 minutes after allowing blood to clot for one hour at 37°C. Sera were stored at −20°C till use. Immunization and blood harvest protocols were followed as per IAEC guideline approval.

Preparation of immunoglobulin fraction from rabbit serum

Sera were first subjected to overnight precipitation at 4°C with ammonium sulphate at 50% saturation and then centrifugation at 5000g for 30 minutes at 4°C. The pellet was resuspended in appropriate volume in binding buffer (0.1 M Tris-Cl pH 8.0) and dialysed using 10 kDa cut off membrane against 1 L of binding buffer for 16 hours at 4°C to remove ammonium sulphate.

Protein-A affinity chromatography

Immunoglobulin rich fraction obtained after dialysis with binding buffer was loaded into a column containing protein A sepharose. Non-specifically bound contaminants were removed by washing with 5 column volumes of binding buffer and then bound IgG were eluted in 1 mL fractions with elution buffer (0.1 M Tris-glycine pH 2.4) into 1.5 mL tubes containing 200 µL of 1 M Tris-Cl pH 9.0 for neutralization. Fractions containing the IgG were checked by measuring OD at 280 nm, then pooled together and concentrated by using centicon YM-50.

Extraction of *A. flavus* spore protein

Spores proteins were extracted by bead beating method (Jenkinson et al., 1981). In brief, about 10^{10} spores were homogenised in 1 mL of extraction buffer [20 mM Tris-Cl (pH 8.0), 1 mM EDTA (pH 8.0) and 2% SDS] with 0.3 mg of glass bead (300 nm size), and then lysed in bead beater for 5 min and then incubated for 5 min in boiling water. Spores debris was pelleted by centrifugation, and the supernatant were analysed in 10% SDS PAGE.

Purification of anti spore protein specific immunoglobulin

Aspergillus flavus spore protein lysate was coupled to cyanogen bromide (CNBr) activated sepharose 4B (GE healthcare) as per manufacturer's protocol. In brief, CNBr activated sepharose CL 4B freeze dried powder (0.5 g) was suspended and subsequently washed with 150 mL of 1 mM HCl. The isolated spore proteins were dialyed in buffer (0.1 M $NaHCO_3$, pH 8.3 with 0.5 M NaCl). 5 mL of dialyed proteins (10 mg/mL) was coupled to activate CNBr sepharose beads by keeping on rotor for overnight at 4°C. The entire material was then transferred to a small glass column, washed and remaining active sites on the beads were blocked by suspending beads in 0.1 M Tris-Cl buffer (pH

8.3) for 2 h at room temperature. The loosely bound antigen was removed by washing with three cycles of alternating pH; each cycle consisting of a wash with acetate buffer (0.1 M, pH 4.0) containing 0.5 M NaCl, followed by wash with Tris-Cl buffer (0.1 M, pH 8.0) containing 0.5 M NaCl. The cupling of antigen in the bead was checked by analyzing an aliquot of antigen coupled beads on 10% SDS-PAGE. Prior to affinity purification, the column was equilibrated with binding buffer (0.1 M Tris-Cl, pH 8.0). Protein A purified immunoglobulin fraction was loaded into the column and unbound antibodies were allowed to pass through and the column was washed with binding buffer until the A 280 of the flow through became negligible. The specific antibody boundto column was eluted with 0.1 M glycine, pH 2.4 and fractions (1ml) were collected in microfuge tubes containing 200 µL of 1 M Tris-Cl pH 9.0 for neutralization. The fractions with A 280 greater than 0.2 (measured using Nanodrop) were pooled together and concentrated by passing through centricon YM- 50 (Millipore) and was checked by 10% SDS PAGE.

Western blot analysis

Western blot analysis was carried out based on protocol suggested by Mahmood and Yang, (2012). *Aspergillus flavus* spore protein lysate was run on 10% SDS PAGE. The proteins were blotted onto nitrocellulose membrane using Mini Trans-Blot ® Cell (Biorad) operated at 20 V for 14 h at room temperature. After the transfer, membrane was blocked with blocking buffer (1XPBS containing 0.05% Tween-20 (PBST) and 3% BSA) for 3 h at room temperature. Membrane was then washed three times using PBST and treated with anti *A. flavus* spore proteins specific affinity purified antibody 1 µg/mL (1:500 dilution) in PBST containing 1% BSA for 3 h at room temperature. Following three times wash with wash buffer; secondary reagent (Protein-A HRP conjugate, GE lifesciences) was added at 1:3000 dilutions in PBST containing 1% BSA and incubated for 2 h at room temperature. After three times wash with wash buffer; colour was developed using Opti-4CN substrate (Biorad) until signal with sufficient intensity was obtained. Reaction was stopped by rinsing the membrane thoroughly with deionised water and then photographed.

Enzyme linked immunosorbent assay (ELISA)

Wells of 96 well ELISA plates (Tarsons, U bottom well) were coated with 100 µL/mL of antigen (spore protein lysate) suspended in coating buffer (0.1 M bicarbonate buffer pH 9.6) for overnight at 4°C. Wells were washed thrice with PBST to remove unbound antigen. Wells were then blocked with 200 µL of blocking buffer (PBST with 3% BSA) for 3 h at room temperature. Wells were washed thrice with washing buffer and 100 µl of 1 µg/mL anti *A. flavus* spore proteins specific affinity purified antibody (1:500 dilutions) in PBST containing 1% BSA was added and incubated at room temperature for 2 h. Following three times wash with wash buffer, 100 µL each of secondary reagents (Protein-A HRP conjugate, GE lifesciences) was added at 1:1000 dilutions and incubated at room temperature for 2 h. Following washing, colour was developed using 100 µL of ABTS substrate (Sigma) along with 1 µL of 30% H_2O_2. Absorbance of the wells was read at 415 nm using microplate ELISA reader (Multiskan spectrum, Thermoscientific).

Results

Identification *A. flavus* fungus

Aspergillus differentiation agar (AFPA) media is a selective media for the enumeration and identification of *A. flavus* and *A. parasiticus* fungus *(*Pitt et al., 1983). The morphological analysis of *A. flavus* showed orange-yellow reverse colony pigmentation on *AFPA* media, colony appeared with globose to sub-globose vesicles and spore showed biseriate seriations, which is a characteristic colony features of *A. flavus* species as described by Klich (2002) (Fig.1). Further, ITS sequence analysis showed 100% query coverage and identity with the repoted ITS sequence for *A. flavus* and confirming its correct identification.

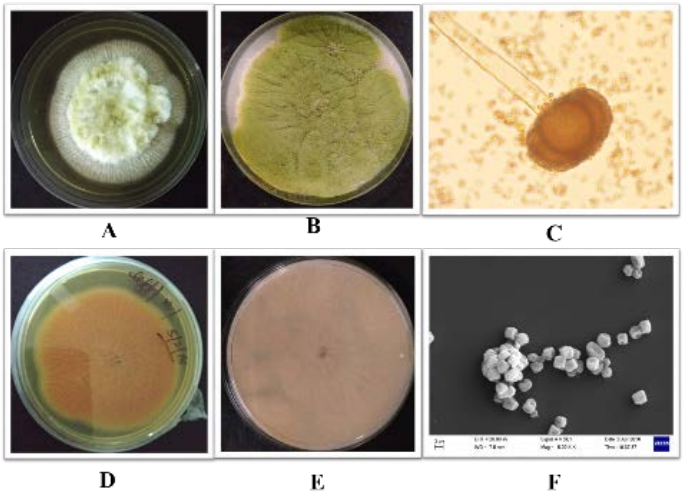

Fig.1. Analysis of *A. flavus* colony characteristics on culture media. A & D' front and reverse view on AFPA. B & E, front and reverse view on coconut media. C, microscopic characteristics of conidial ornamentation on hyphae. F, Spore morphology analysis in SEM.

Purification of anti spore protein Immunoglobulin

SDS PAGE analysis of Immunoglobulin protein fractions obtain after purification through protein A affinity column and through antigen affinity (spore protein) conjugated CNBr column also showed two sharp bands at 50 kDa and 25 kDa corresponding to the heavy and light chains of Immunoglobulin respectively indicating the successful purification of anti spore protein immunolo antibodies (Fig.2). The crude serum protein percipited using 50% ammonium sulphate solution. It showed smear of protein band through out the lane which indicated precipitation of several other proteins along with immunoglobulin. Futher purification through potein A column discarded other proteins and allowed purification of immunoglobulin proteins. The eluted immunoglobulin fraction was passed though the antigen conjugated CNBr activated sepharose column and purification of anti spore proteins antibodies was done.

Analysis of anti spore specific antibody for detection of A. *flavus*

The lysate of spore proteins run on 10% SDS PAGE showed several protein bands ranging from 10 kDa to 200 kDa (Fig.3A). The same amount of proteins was run in 10% SDS PAGE and trans bloted on nitrocellulose membrane. The blot showed two prominent proteins bands of around 76 kDa and 100 kDa (Fig.3B.).

Detection of *A. flavus* fungus by ELISA

The sensitivity of antibody for detection of spore protein was determined by indirect ELISA method. The protein concentration ranging form 10 µg /mL to 200 µg /mL in carbonte buffer were used for detection of sensitivity of antibody. The minimal detectable concentration of antigen was 20 µg /mL.

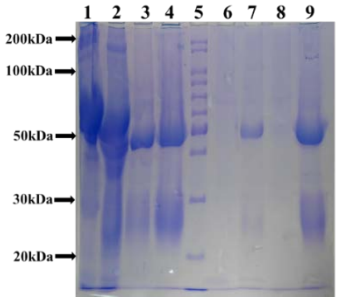

Fig.2. SDS PAGE analysis of IgG purifed from protein A sepharose column. Lane 1, Serum proteins; lane 2, Ammonium sulphate precipitated IgG; lane 3, IgG elute from column; lane 4, Concentrated IgG; lane 5, Protein marker; lane 6, filtrate collected from 50 kDa centricon; lane 7, antigen affinity purified concentrated IgG; lane 8, filtrate collected from 50 kDa centricon of affinity purified IgG; lane 9, unbound IgG from antigen affinty column.

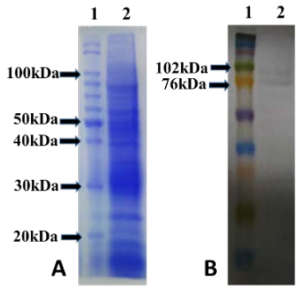

Fig.3. Western blot of *A. flavus* spore protein. (A) comassiae blue stained gel, lane 1, protein ladder, lane 2, spore protein lysate. (B) corresponding western blot profiles of lanes 1-2.

Discussion

India falls in tropical region, where fungal infestation is widely prevelent in pre and post harvested crops. It is estimated that approximately 25–50% of harvested food and feed gets contaminated with mycotoxins (Abdin et al., 2010). In present study, *A. flavus* species was isolated from stored wheat grains, and characterized by morphological and molecular method. Zulkifli and Zakaria, (2017) have suggested that to identify a fungus at species level, both morphological and molecular identification should be applied because for some species, morphological characteristics may be similar. Internal transcribed spacer (ITS) region of ribosomal gene is a universal barcode for molecular identification of fungal species (Pryce et al., 2003). Combination of morphological identification and sequencing of ITS can reliably identify Aspergillus isolates to species level.

Aspergillus flavus can produce aflatoxins, which is a toxic and carcinogenic secondary metabolite, and their contamination in food grains can adversly affect its quality and usability (Sun et al., 2016). Fungus produces sexual/asexual spores, in order to survive for long term in adverse physiological and environmental conditions. It is a key attribute for fungal reproduction, persistence, and dispersal. Therefore, spore would be a suitable antigen for immunological detection of fungal contaminants in stored grains. Polyclonal antibodies were raised against whole inactivated *A. flavus* spore in rabbit and purified through protein-A column followed by antigen affinity chromatography. Resulting antibody showed high specificity against *A. flavus* fungus as seen from the results of western blot where strong positive reaction of antibodies against two spore proteins were visualized. Further, these antibodies were again tested for sensitive ELISA for detection of *A. flavus*. These polyclonal antibodies can be used for the development of a specific and sensitive technique like dot blot ELISA for monitoring fungal contamination level in stored grains.

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Smallholder farmers' perceptions of aflatoxins in maize in kamuli district, Uganda

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Extended Abstract

Keywords: postharvest, harmful effects, mold, outreach, education

Aflatoxins are a family of highly toxic and carcinogenic compounds produced by fungi commonly found on maize. Aflatoxins have been estimated to be widespread in maize in Africa (Wagacha and Muthomi, 2008). Consumption of aflatoxins in foods is associated with liver cancer in adults and stunted growth and development in children. Studies have shown that over 50% of maize in Ugandan markets contain over 10 ppb aflatoxins, the safety limit set by Uganda National Bureau of Standards. Present in maize fields, these fungi (molds) continue to grow on maize when it is insufficiently dried and then stored, thereby increasing aflatoxin levels. Maize is an important staple crop for smallholder farmers. They often have difficulty properly drying and storing maize and thus face the risk of aflatoxin consumption. Previous published studies revealed that many smallholder farmers do not know what aflatoxin is nor the risks they face from it (e.g., Magembe et al., 2016).

Iowa State University Uganda Program (ISU-UP) works in smallholder farmer communities in the Kamuli district of Uganda to improve peoples' health, nutrition and rural livelihoods (www.csrl.cals.iastate.edu). It operates eight Nutrition Education Centers (NECs) where pregnant women and mothers of nutritionally challenged children within their first 1000 days of life can