JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Sept. 2017. Vol. **11**(3), p. 1251-1258

Molecular Characterization and Bioinformatics Analysis of ACT-toxin Produced by *Alternaria* spp. Isolated from Corn and Rice in Saudi Arabia

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http://dx.doi.org/10.22207/JPAM.11.3.03

(Received: 29 July 2017; accepted: 23 September 2017)

The Current study revealed the natural occurrence of toxigenic fungi and mycotoxins production in grains in Saudi Arabia. Samples of yellow corn, white rice and red corn grains were collected from different local markets. Three fungal isolates were isolated from the examined corn grains using PDA media. The toxigenic *Alternaria* spp. was the most prominent fungi in yellow corn grains, white rice and red corn grains. Three *Alternaria* spp. isolated were identified using molecular characterization of *ACTTS* gene. DNA genome of the three *Alternaria* spp isolates (namely AWR; AYC and ARC which corresponds to isolates from white rice, yellow corn, red corn) was used as a template for PCR to amplify *ACTTS* gene. Partially sequenced *ACTTS* gene was amplified using a specific primer set to confirm its identity, phylogenetic relationships between the three isolates as well as determination of the corresponding antigenic determinants. The epitope prediction analysis demonstrated that there were 5, 6 and 5 epitopes whose score were above 0.90 in AWR, AYC and ARC, respectively. Interestingly, there were great variations in the epitope sequences among the three isolates except for the epitope, VYGASTATGTLAVQ. This work led to molecular identification of three *Alternaria* spp. using *ACTTS* gene and the unique antigenic determinants that could be used for design of a broad spectrum antibody for rapid detection of *Alternaria* spp. in foods.

Keywords: Mycotoxins, *Alternaria*, *ACTTS* gene , PCR , antigenic determinants.

Several fungi attack the corn grains during harvest and storage. While more than 25 different fungi species known to invade stored grains (Duan et al., 2007), some species such as Aspergillus, *Fusarium, Penicillum* are responsible for most spoilage and germ damage during storage (Pitt, 2000a). They cause reduction in baking quality, nutritive values, produce undesirable odors, color and changes appearance of stored food grade seeds and decrease germination ability and total decay (Castillo et al., 2004). Besides their mycotoxins, they are considered as health hazard for man and animals, render products unacceptable for edible purposes or lower their market grade (Pitt, 2000b; Richard et al., 2007). Moreover, fungal infestation of seed coat decreases viability of seeds, or may cause abnormal seedlings (Selcuk et al., 2008). This has been demonstrated by isolation of fungi from seeds collected before seed set. Many of these fungi have no negative impact on seeds but there also many saprophytic and pathogenic fungi commonly isolated from seeds (Garcez et al., 2000) .The knowledge on frugal seed decay and its importance for plant demographic and community processes is quite limited (Blaney and kotanen, 2001). Fungal

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genera, such as *Aspergillus* spp*; Fusarium* spp*; Penicillium* spp*; Alternaria* spp*;* and *Epicoccum* spp. were isolated from same seeds of grains, beans, cowpea, peas, and cocoa (Rodrigues and Menezes, 2005; Sánchez-Hervás et al., 2008; Weder, 2002). In Saudi Arabia, very little information exists with respect to its natural contamination with toxigenic fungi and mycotoxins. Aflatoxin (s) were detected in some *Aspergillus* isolates while fumonisin was detected in some *Fusarium* isolates (Ibrahim et al., 1998) . Some pathotypes of *Alternaria Alternata* produces host selective ACT-toxin for which several open reading frames designated as *ACTTS* are implicated for its synthesis (Ajiro et al., 2010). Determination of *ACTTS* gene in *Alternaria* and unraveling its characteristics are important because it is involved in ACT-toxin production and pathogenicity.

The aim of the current study is the molecular identification of toxigenic Alternaria contaminating some grains and protein structural analysis depicted from the gene(s) responsible for toxin biosynthesis.

MATERIALS AND METHODS

Grains samples

One hundred fifty grains corn (yellow and red corn grains) and rice (short and long white rice) were collected from different area of Saudi Arabia (Riyadh, Hail, Qasim, Asir,Tabuk, Jizan, Jouf, Jeddah and Dammam), where collected from storage markets and houses . The collected grains were randomly and its weight between 0.5 - 1 kg of each grain in cleans and dries packaging.

Isolation of mycotoxigenic *Alternaria* spp.

Agar plate and blotter tests were used to isolate *Alternaria* spp. as described by Neergaard (1977). Grains were divided into two groups, the first group was disinfected with sodium hypochlorite 1% for 2 min and the second group was non-disinfected. All grains were washed several times by sterilized water, and then dried between sterilized filter papers. The half of each group was plated on potato dextrose agar (PDA). All dishes were incubated for 5 to7 days at 25 °C.

Purification and identification of *Alternaria* spp.

Single colony was transferred and purified by hypha tip technique onto PDA medium in the presence of streptomycin (50 mg /ml). The developing fungi were prepared for molecular identification using primers specific for the *ACTTS* gene.

Molecular identification of *ACTTS* gene

The molecular identification of the *ACTTS* gene was carried ou**t** by PCR and sequencing of amplicons.

Isolation of DNA genome

The mycelium mass of *Alternaria* spp isolates grown on PDA broth medium was harvested by centrifugation at 6000 rpm for 10 min. The pellets were washed twice by PBS buffer and stored at 20^0C. Total DNA of the three isolates was isolated using lysozyme – dodecyl sulfate lysis method as described by Leach, et al (1990).

Amplification and purification of *ACTTS* gene

Specific PCR reactions were conducted to assess the presence of *ACTTS* gene. The primers used in this study are provided in a supplementary table. The PCR amplification conditions included initial denaturation at 94 °C for 5min then 35 cycles at 94° C for 30 s, 55° C for 60 s followed by extension step at 72° C for 90 s. and a final extension at 72° C for 7 min. The amplification reaction was performed by thermal cycler (COT Thermocycler model 1105). Purification of PCR product was detected by electrophoresis using agarose 1.5% in 1x TAE buffer and staining with ethidium bromide (Sambrook et al., 1989). The resultant fragment of *ACTTS* gene was excised from the gel and purified using a QIA quick gel extraction kit (Qiagen, Berlin, Germany).

DNA sequencing

The purified PCR products were prepared for Sanger sequencing technology using DNA sequencer technique (Sigma, central lab, PNU, KSA). DNA sequences of *Alternaria* isolates were aligned using Bio Edit software version 7(www. mbio-ncus. edu/bio. edit) and were compared of the often accessions of *Alternaria* spp. available in the NCBI data base using BLAST algorithm to identify closely related sequences (http/www.ncbi. nih.gov). Dendrogram were constructed by using un-weighed pair Group method with Arithmetic (UPGMA) on Gen bank.

Epitope prediction and antigenicity

The primary amino acids sequence of the *ACTTS* protein was evaluated from the corresponding nucleotide sequence using MEGA 6.0 software. The linear B-cell epitopes in the

primary amino acid sequence of the coat protein was performed using BCPREDS server with default parameters (http://ailab.cs.iastate.edu/bcpreds/) which implements a support vector machine (SVM) and the subsequence kernel method (El-Manzalawy et al., 2008a). Flexible length linear B-cell epitopes were predicted using FBCPred (El-Manzalawy et al., 2008b) method with a specificity cut-off; 75 %.

The antigenicity of each amino acid residue in the primary protein sequence was determined using a semi-empirical method (Kolaskar and Tongaonkar, 1990) which makes use of physicochemical properties of each amino acid and their frequencies of occurrence in experimentally known segmental epitopes.

RESULTS

Three *Alternaria* isolated from tested grains by PDA method was purified by single spore and hypha tip on PDA slant medium. The *Alternaria* isolates were selected for molecular identification using *ACTTS* gene sequencing. Three *Alternaria* isolates represented grains from yellow corn, white rice and red corn and designated as *Alternaria* AYC, AWR and ARC, respectively.

Molecular characterization of *ACTTS* gene

Total DNA was extracted from *Alternaria* spp. AWR; AYC and ARC infected grains. *ACTTS* gene of three *Alternaria* spp isolate AWR; AYC and ARC was amplified from isolated DNA of mycelium using PCR reaction mixture and specific primer sets. PCR amplicons were allowed for sequencing reaction through cycle sequencing method. The DNA Amplicons returned as electropherogram Files. Electropherogram showed distinct peaks for each base cell as well as high Q values for each Cell. Sequences obtained for each primer for each isolate had sufficient overlap between them and used to form one continuous sequence (Coting). The nucleotide partial sequence of *ACTTS* gene in the three isolates was compared with published isolates on GenBank. The sequence homology revealed that the gene of interest was *ACTTS* gene (coding for enoyl reductase) and the test fungal isolates were *A. alternata* isolates. A multiple sequence alignment was constructed using Clustal W software between the three studied isolates. The alignment showed many conserved regions in all sequences as well as distinguished the heterogeneity positions among the aligned sequences. Phylogenetic analysis was performed by construction of phylogenetic tree using a neighbor joining method to unravel the relationships among all *Alternaria* isolates (Fig. 2). The phylogenetic tree resulted in two clades in which AYC (yellow corn isolate) and AWR (white rice isolate) were in the same cluster whilst ARC (red corn isolate) was separate in a different cluster. Thus, the molecular identification based on sequence homology of the *ACTTS* gene confirmed the identity and phylogeny of the studied three *Alternaria* isolates.

Detection of epitope sequences of the *Alternaria ACTTS* gene

For prediction of the linear B-cell epitopes in the *ACTTS* protein primary amino acids sequence, BCPREDS server was used. Five to six epitopes, whose sequence length was 14 residues, were retrieved in the amino acid sequences of the three *Alternaria* isolates (Table 1). The highest epitope according to specificity was AMGAKGGKYTALLP, LEKEKIKTHPVGVR and LEKEKIKTHPVGVR in the *ACTTS* in AWR, AYC and ARC, respectively. The epitope prediction analysis demonstrated that there were 5, 6 and 5 epitopes whose score were above 0.90 in AWR, AYC and ARC, respectively. Interestingly, there were great variations in the epitope sequences among the three isolates except for the epitope, VYGASTATGTLAVQ which was found to be conserved among all. The predicted epitopes were displayed and highlighted (in red) with their positions on the amino acids sequence as shown in Figure (2).

The antigenicity profile of the amino acid residues of the *ACTTS* protein demonstrated the residue of high frequency to be part in the genetic determinant (fig. 4). The highest residues above the threshold value were numerous such as Valine (V), Leucine (L), Isoleucine (I), serine (S), Cytosine (C), Aspartic acid (D) , glutamine (Q) and glutamic acid (E) with antigenicity score and spanning the region, 281- 293, 218-230, 280-290 in the primary protein sequence of ARC, AWR and AYC, respectively. These residues with high frequencies of occurrences in antigenic determinants were highlighted (yellow) in the antigenicity profile

(fig. 3). Figure 3 also show the variability in the positions and types of amino acid residues with high antigenic frequency.

DISCUSSION

The emergence of toxigenic fungi on small grains has a negative impact on the safety and quality of feed and food. The genus *Alternaria* includes cosmopolitan and ubiquitous mold fungi in which saprobes and plant pathogens are many (Thomma, 2003). *Alternaria* species causes yield losses in processing and production (Vukovi, 2012). Being able to grow at low temperature, *Alternaria* spp. are responsible for spoilage of food commodities during transport and storage (Barkai-Golan, 2008; Dall'Asta et al., 2014). In addition, reduction in nutritive value, insipidness and discoloration are other problems resulted from contamination of grains by *Alternaria* (Kosiak et al., 2004). In addition to economic losses, many species are well known mycotoxins producers with various toxicological properties and high risks for human and animal health (Logrieco et al., 2009). Rapid and accurate identification of *Alternaria* and / or their metabolites are mandatory for the implementation of preventive measures in the whole food production system. The molecular characterization of the three *Alternaria* spp. isolated from small grains (yellow corn, white rice and red corn) using the mycotoxins gene was performed and the *ACTTS* gene sequence analysis allowed for coupled identification and mycotoxins screening in the three *Alternaria* isolates. Mycotoxins-producing fungi were isolated from sorghum grains from Saudia Arabia before (Mahmoud et al., 2013; Yassin et al., 2010). Following the molecular identification of *Alternaria* spp., B-cell epitopes in the *ACTTS* gene were predicted. The characterization of B-cell epitopes using computational tools is highly advantageous for the synthesis of specific antibodies for rapid detection of microbial pathogens in their environments. The epitopes prediction saves labor and time for validation experiments. The identification of epitopes plays a crucial role in the vaccine design, immunodiagnostic testing and antibody production (Sette and Fikes, 2003). In this study, BCPREDS server was used to predict epitopes found in the primary amino acids sequence of *ACTTS* protein.

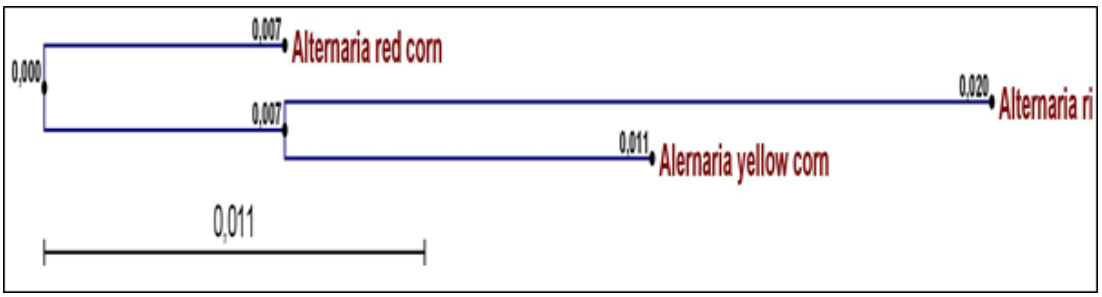

Fig. 1. Phylogeny of the three studied *Alternaria* isolates (*A. Alternata* AYC, *A. alternata* AWR and A. alternata ARC) isolated from yellow corn, white rice and red corn

Table 1. Flexible length predictions of epitopes in the amino acids sequence of *ACTTS* protein of the three studied *Alternaria* isolates

No.	Epitope/AWR	Score/ AWR	Epitope/AYC	Score/ AYC	Epitope/ ARC	Score/ ARC
1	AMGAKGGKYTALLP	0.996	LEKEKIKTHPVGVR	1	LEKEKIKTHPVGVR	1
2	GPHATPGSILGCDY	0.992	AMGAKGGKYTALLP	0.996	WPAAMGAKGGKYTA	0.999
3	LEKERSMTHPVGVR	0.991	WKMLYGPHATPGSI	0.977	GPHATPGSILGCDY	0.992
4	VYGASTATGTLAVQ	0.973	VYGASTATGTLAVQ	0.973	VYGASTATGTLAVQ	0.973
5	FAFRECPYNHDEGG	0.958	FDYRNPECGAKIRT	0.938	IKNFPRSDVKVTTI	0.911
6	-	-	PANQKDFEFSAFWK	0.901	-	-

BCPREDS proved high efficiency to predict linear B-cell epitopes in SARS-CoV S protein (El-Manzalawy et al., 2008). There was variability in the sequence and numbers of epitopes among the three toxin proteins analyzed. Here, a fixed length of epitopes (14 residues) was observed. The epitope, VYGASTATGTLAVQ, was found to be common between all isolates suggesting its exploitation for design of a specific antibody to be used for rapid detection of different *Alternaria* species in small grains. The highly frequent residues with high antigenicity profiles such as Valine, Leucine, Isoleucine, Aspartic acid, Glutamine and Glutamic acid are mostly hydrophobic. The occurrence of

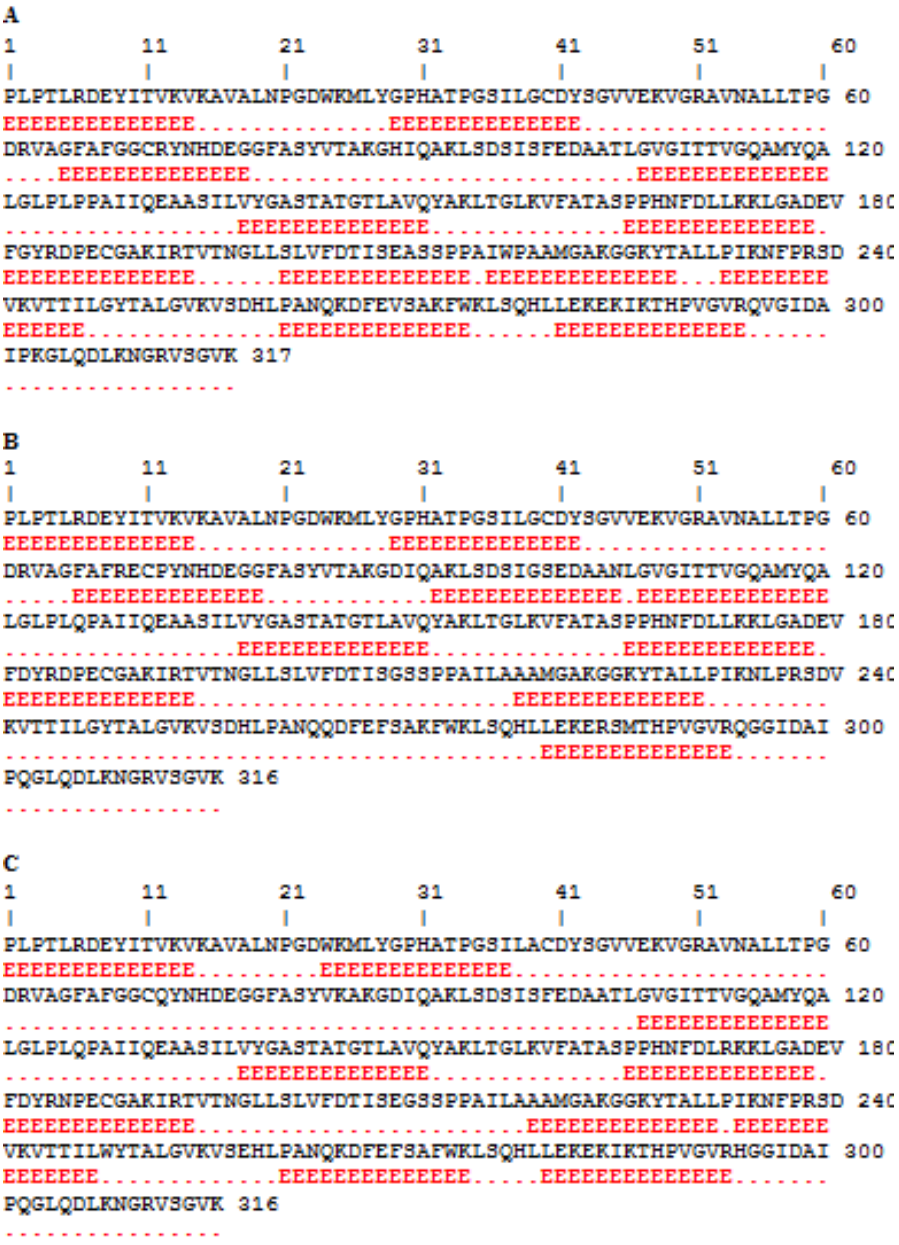

Fig. 2. Amino acid residues of *ACTTS* protein in ARC (A), AWR (B) and ARC (C) showing predicted as epitopes (Red) that are highlighted

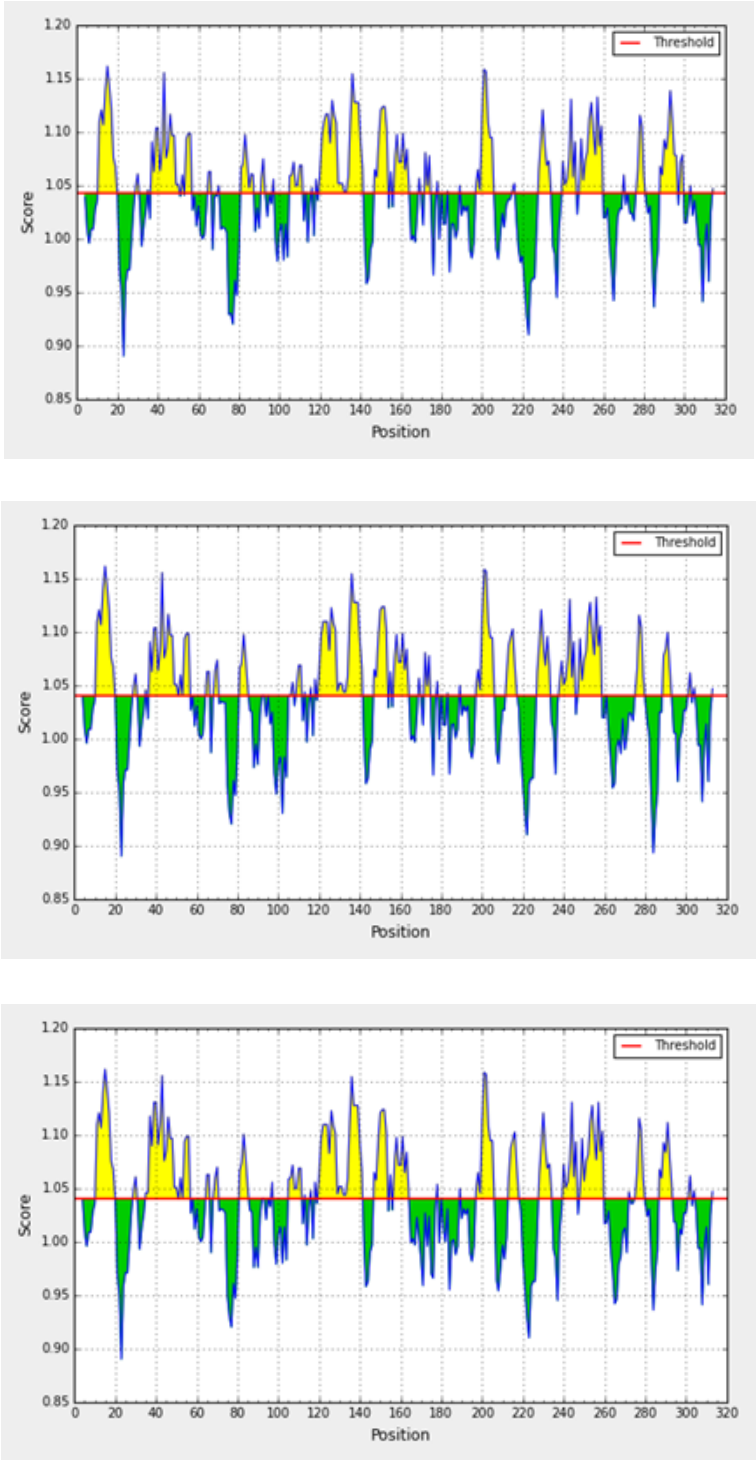

Fig. 3. Kolaskar and Tongaonkar antigenicity scale for prediction of antigenic determinants in *ACTTS* protein in ARC (A), AWR (B) and AYC (C). Amino acid residues of high frequencies in epitopes are distinguished (yellow)

hydrophobic residues in epitopes is frequent and do have a hierarchy signature (Aftabuddin and Kundu, 2007; Mine and Zhang, 2002). Epitope prediction has many implications in pathogen detection and differentiation applications.

The consideration of occurrence of *Alternaria* spp. on small grains is important in risk assessment of mycotoxins and setting up preventive measures proactively.

ACKNOWLEDGEMENT

The authors are grateful to Deanship of Scientific Research; PNU for financial support. And thanks for all technicians in Research Labe, Biology department, PNU.

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